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14. ABSTRACT
The purpose of the project was test the prediction that interventions that reduce the function of sodium ion channel Nav1.8 (primarily expressed in nociceptive primary afferent neurons) ameliorate reflex hypersensitivity and pathological pain-related motivational/cognitive alterations caused by traumatic spinal cord injury (SCI). Initial findings were published in a major paper showing that antisense knockdown of Nav1.8 eliminates SCI-induced spontaneous activity (SA) in nociceptors, reverses mechanical and heat hypersensitivity of withdrawal reflexes, and ameliorates spontaneous pain. Extending these findings to pharmacological inhibitors of Nav1.8, intraperitoneal delivery of a selective Nav1.8 antagonist, A-803467, showed modest effects on heat hypersensitivity, mechanical hypersensitivity, spontaneous pain, and anxiety, while the nonspecific Nav1.8 antagonist, ambroxol, was ineffective. Preliminary results with intrathecal delivery of A-803467 are exciting because of the implication that ongoing Nav1.8 function in nociceptor somata with SA is required for spontaneous and evoked pain. Other findings led to a major paper showing that blockade of the cAMP-PKA signaling pathway, which enhances Nav1.8 activity, eliminates SCI-induced nociceptor SA. This project has set the stage for testing the predicted effects of selectively targeting somal (rather than systemic) Nav1.8 on the maintenance of pain, dysesthesia, spasticity, and anxiety after SCI.

15. SUBJECT TERMS
Spinal cord injury, chronic pain, spontaneous pain, evoked pain, anxiety, primary nociceptors, Nav1.8, hyperreflexia

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1. INTRODUCTION

The purpose of this project was to test a novel approach to treating chronic pain and other complications of spinal cord injury (SCI) using a preclinical rat model. Over 40,000 veterans have SCI, as well as many active members of the armed services, and a majority of these people endure intractable pain and potentially related chronic problems such as anxiety and gastrointestinal dysfunction for the rest of their lives. Most investigators have assumed that the critical mechanisms driving SCI pain are located within the central nervous system (CNS) and involve direct effects of the injury and/or associated neuroinflammation on pain pathways (Finnerup and Baastrup, 2012; Walters, 2012; Walters, 2014). Early evidence that primary sensory neurons, and especially primary nociceptors, are involved in neuropathic SCI pain came from observations of enhanced nociceptor growth after SCI (Bedi et al., 2012). Primary nociceptors are the first neurons within pain pathways and thus their electrical activity leads to the conscious sensation of pain as well associated reflex responses. These sensory neurons are specialized for the detection of bodily injury and inflammation and are normally electrically silent, firing action potentials only when their peripheral branches are activated by stimuli that can produce pain (fortunately, an infrequent occurrence for most people). This award enabled rigorous tests of our prediction that prominent aspects of chronic pain and hypersensitivity caused by SCI can be ameliorated effectively by interventions that selectively block spontaneous electrical activity in primary nociceptors. Six years ago we reported (Bedi et al., 2010) the unexpected discovery that primary nociceptors in rats that have received a contusive spinal injury (controlled experimental bruising of the spinal cord) months earlier continuously fire action potentials without any extrinsic stimulation ("spontaneous activity," SA), even when the recorded nociceptor is removed from the body and isolated from all other cells. Electrical activity in any nociceptor would be expected to excite pain pathways and thereby promote pain sensations, and so it was not surprising to find that this chronic nociceptor SA was closely correlated with behavioral measures of pain; animals exhibiting pain showed a high incidence of nociceptor SA whereas apparently pain-free animals did not. More direct evidence that activity in primary nociceptors helps to maintain SCI pain came from our finding that antisense knockdown of TRPV1 channels or pharmacological blockade of TRPV1 channels -- which are expressed most abundantly in nociceptors -- reduced SA after SCI and caused a dramatic reversal of reflex hypersensitivity (Wu et al., 2013). Importantly, the nociceptors exhibiting SA after SCI possess an ion channel, Nav1.8, that in the nervous system is only expressed by primary sensory neurons, and primarily in nociceptors (Shields et al., 2012). We found that a drug (A-803467) that selectively blocks Nav1.8 channels blocks SA in nociceptors after SCI. These discoveries led directly to the hypothesis that Nav1.8 function is critical to maintenance of pain and related consequences of SCI, and thus to the experiments in this project. We tested the prediction that interventions that reduce Nav1.8 function -- specifically antisense knockdown of Nav1.8 expression and inhibition of Nav1.8 channels using two different drugs – would reduce chronic pain and other debilitating behavioral effects (as well as SA and hyperexcitability in nociceptors) after SCI. To model chronic dysfunction after SCI, animals were tested 4 to 12 weeks after injury. A novel and potentially important part of our experimental design was to apply operant measures of ongoing, spontaneous pain, evoked pain, and other emotional consequences of SCI. These provide more relevant models of pain and suffering after SCI than the hyperreflexia measures that almost all previous studies of SCI pain relied on. Our experiments confirmed some of our major predictions, but have also revealed unexpected behavioral consequences of SCI that complicate the measurement of pain and hyperreflexia, while suggesting an even more profound than anticipated role for nociceptor SA in persistent suffering produced by SCI.

2. KEYWORDS

spinal cord injury, chronic pain, spontaneous pain, evoked pain, anxiety, primary nociceptor, spontaneous activity, Nav1.8, hyperreflexia

3. OVERALL PROJECT SUMMARY

Major objectives (aims) of the project

Aim 1 (Tasks 1b, 1c): Test the hypothesis that chronic reflex hypersensitivity in a rat contusive SCI model is reduced by blocking spontaneous activity (SA) in nociceptors via reduction in Nav1.8 activity achieved by either knocking down Nav1.8 channel expression or by applying a highly specific Nav1.8 blocker, A-803467. 95% completed.

Aim 2 (Tasks 1d, 1e): Test the same hypothesis by seeing if a less specific but much less costly Nav1.8 blocker, ambroxol, can be used for both brief and prolonged attenuation of behavioral hypersensitivity after SCI (Tasks 1d, 1e). 100% completed.

Aim 3 (Task 1f): Test the prediction that chronic visceral hypersensitivity after SCI can be reduced by decreasing the activity of Nav1.8 channels. This task was begun with Dr. Hongzhen Hu, a collaborator in the department with expertise in visceral pain testing, but had to be abandoned when Dr. Hu left the institution and because it was found that repeated testing of the same animals with multiple types of pain testing caused excessive stress.

Aim 4 (Tasks 2a-2e): Show that decreasing Nav1.8 activity or expression reduces evoked and spontaneous motivational and cognitive features of pain-related behavior after SCI. 80% completed.

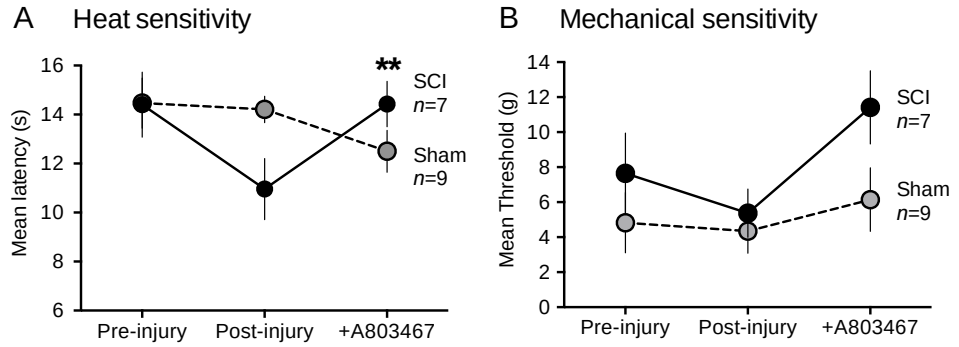
Accomplishments under each task

Task 1a - Institutional and DOD animal use approvals. Accomplished.

Task 1b – Investigate reflex hypersensitivity effects of knocking down Nav1.8 expression. We published a paper (Yang et al., 2014) documenting our findings from work completed in Years 1 and 2 of this award on the suppression of SCI-induced hyperreflexia by Nav1.8 knockdown.

Task 1c – Investigate reflex hypersensitivity effects of selectively blocking Nav1.8 activity with low- or high doses of A-803467.

Fig.1

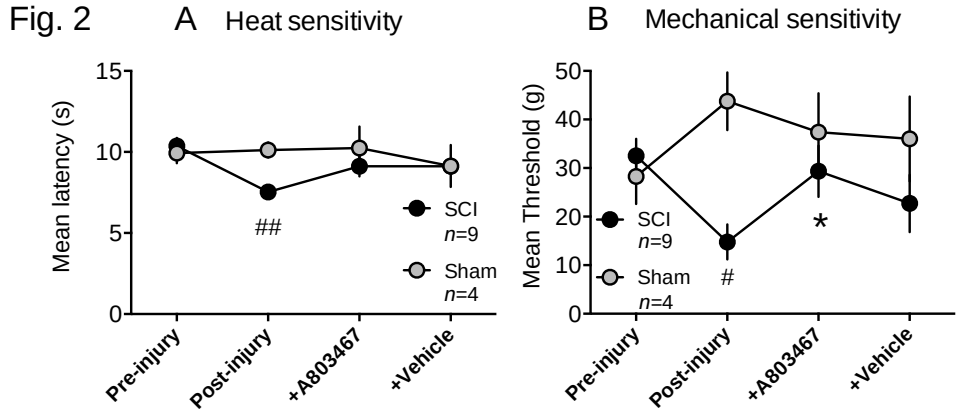


We have continued to test whether delivery of had been the most selective Nav1.8 antagonist available, A-803467, reduces SCI-induced hyperreflexia. Because of low bioavailability, this drug is very expensive to use for whole-animal studies; even after a discount of nearly 70% that we negotiated with Selleckchem for volume purchases, it costs about $60 per i.p. injection per rat to test behavioral effects of A-803467. Consequently, we limited this Aim to testing the acute effects of a single injection of the drug, even though prolonged or repeated application would be expected to produce effects closer to that produced by antisense knockdown. We found that a relatively low dose, 30 mg/kg (Jarvis et al., 2007), had very weak effects, if any, so we focused our studies on the higher dose, 100 mg/kg. The results of our first study are summarized in Fig.1. The effect on hypersensitivity to radiant heat stimulation of the hindpaws seemed clear (Fig.1A). A-803467 injection 20 min before testing caused a significant reversal of the withdrawal latency that had been reduced by SCI when tested ~1 week earlier (more than 1 month after injury). This result suggested that SCI-induced hypersensitivity to heat stimulation has a moment-to-

moment dependence upon electrical activity requiring Nav1.8 channels, and thus encouraged further investigation into the potential use of Nav1.8 antagonists for the treatment of SCI pain. However, the effects of A-803467 on mechanical sensitivity in the same animals (Fig.1B) were more difficult to interpret, and led us to consider the possibility that the drug effects were variable because the degree of pain produced by our SCI protocol at the time had become more variable.

Surgical and testing procedures were then improved by sending Max Odem and Dr. Alexis Bavencoffe to the Spinal Cord Injury Research Training Program at Ohio State University (OSU) in May of 2015. On the basis of information from the Ohio State course and input from SCI surgeons Drs. Qing Yang and Juan Herrera in this institution, and veterinarians from our animal facility, we improved the effectiveness of our SCI procedures and greatly reduced mortality and morbidity caused by sub-optimal design and implementation. In contrast to our high attrition rates during the first two years, none of the many animals given SCI by Max died or developed infections, and <5% had to be discarded because of "bad hits" on the spinal cord (glancing blows). This advance reflects improved sterile procedures and a more sophisticated understanding of how to control the Infinite Horizon device.

Testing procedures were improved in several ways. First, we found that a male tester inadvertently caused more stress to the rats than did the female testers. Reinforcing this conclusion was publication of an important paper documenting stress-induced analgesia in rats and mice produced by olfactory cues from male experimenters (or even their clothing) several feet away from the rodents (Sorge et al., 2014). During the 6-month EWOF extension we hired an experienced female tester (paid by the PI's endowment funds) and she conducted the experiments shown in Fig. 2. Additional changes were adopted from the Ohio State procedures: we used a larger number of von Frey filaments for mechanical testing (adding stiffer filaments) and modified the "up-down" procedure so that all animals received 10 applications of the filaments. These changes probably explain the noticeably higher mechanical thresholds in Fig. 2 than in Fig. 1. In addition, we realized that unconscious tester bias was impossible to prevent in post-injury tests comparing SCI to sham-treated animals. Therefore we tested on two days after the post-injury test, once with the drug of interest (A-803467 in Fig. 2) and once with vehicle, with the order counterbalanced.



The new procedures revealed once again that SCI causes heat and mechanical hypersensitivity (Fig.2, # $p<0.5$, ## $p<0.01$ versus pre-injury). In addition, mechanical (and possibly mechanical hypersensitivity was reversed by i.p. injection of Nav1.8 antagonist A-803467 (* $p<0.05$). However, the effects of vehicle treatment were not significantly different from A-803467 treatment. One possible explanation is that even very careful, highly experienced animal testers may be influenced by unconscious expectations (they can distinguish SCI from sham animals, but not drug treatment from vehicle treatment when blinded). Another possibility is that prolonged i.p. injection of the highly viscous vehicle for A-803467 (which is very difficult to inject and has poor bioavailability) was sufficiently stressful as to produce analgesic effects. In either case, we cannot yet conclude that transient pharmacological blockade of Nav1.8 reverses SCI-induced reflex hypersensitivity. These complications provided additional motivation to find operant tests for hypersensitivity and evoked pain that are automated and not subject to tester bias, and to explore intrathecal (i.t.) application of Nav1.8 antagonists (see Tasks 2a and 2c).

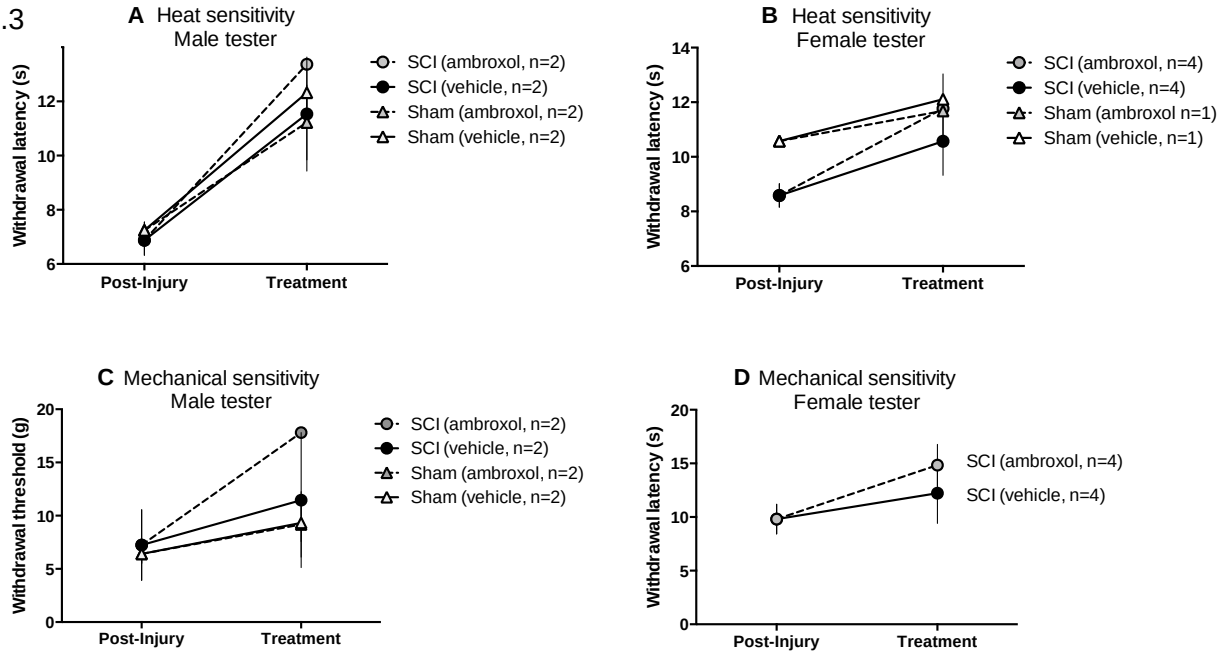
We also examined other antagonists that are likely to reduce Nav1.8 function, albeit indirectly. These studies made use of animals that had been used for the behavioral pharmacological studies in this SOW. Cyclic AMP via PKA enhances Nav1.8 activity. Alexis Bavencoffe, in collaboration with Dr. Carmen Dessauer in this department, found that blocking cAMP-PKA signaling in nociceptors blocks SCI-induced spontaneous activity (SA) in these neurons, in part through a potential reduction in Nav1.8 activity. SA recorded under current clamp in nociceptors dissociated from SCI animals 4-8 weeks after injury was blocked by either pretreatment or superfusion after starting patch recording with several PKA blockers and PKA-AKAP disruptors, including Rp-cAMPS, stHt31, STAD2, and H-89. These results have been combined with biochemical and molecular data from the Dessauer lab and published in the *Journal of Neuroscience* (Bavencoffe et al., 2016).

Task 1d – Investigate behavioral hypersensitivity effects of blocking Nav1.8 activity with single low and high doses of ambroxol.

We attempted to test the prediction that the less specific and less costly Nav1.8 blocker, ambroxol, can be used for both brief (Task 1d) and prolonged (Task 1e) attenuation of SCI-induced reflex hypersensitivity. We initially planned to use oral gavage as the delivery procedure. However, our finding that antisense knockdown of Nav1.8 prevented spontaneous pain after SCI (Yang et al., 2014), as indicated by blocking of conditioned place preference (CPP), encouraged us to use a delivery route that would be better suited to the CPP procedure. In particular, if ambroxol was to be used as an effective analgesic in the conditioning of place preference (Task 2), it is necessary that the onset of ambroxol's analgesic effect be rapid so that it can be timed to coincide with placement of the animal in the chamber that the animal will learn is a refuge from spontaneous pain. Because oral gavage produces a slow build-up of systemic drug levels, we decided to see if i.p. injection of ambroxol or its vehicle had any analgesic effect or other effects.

We first examined the effect of a single i.p. injection of ambroxol on the sensitivity to heat or mechanical stimulation after SCI and found complex results. As shown in Fig.3, one complication was that the results depended upon whether testing was performed by a male (Max Odem) or female (Robyn Crook) tester. The male tester found no differences between sham and SCI treatment post-injury in either heat or mechanical sensitivity (Fig. 3A and C). The female tester found an apparent reduction of

Fig.3

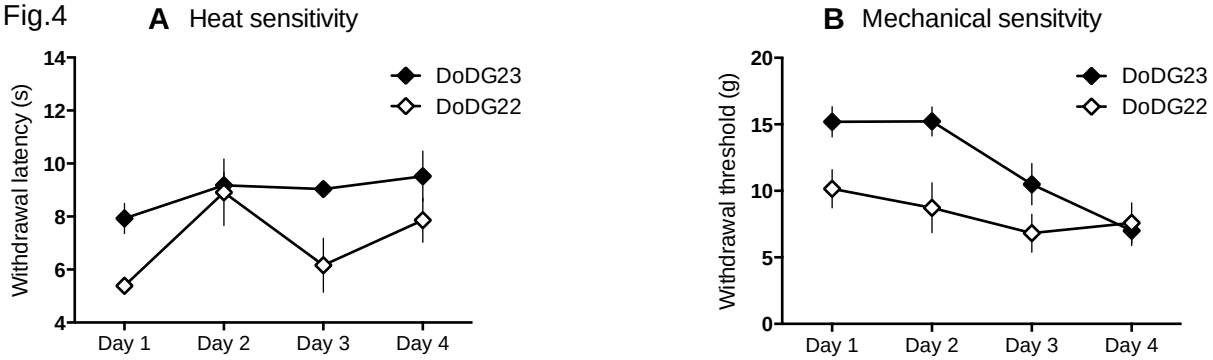


latency (increased sensitivity) during heat stimulation post-injury compared to the 2 sham animals that were tested (1 in each sham group) (Fig.3B). In addition, all post-injury responses measured by the male tester tended to be sensitized compared to those measured by the female tester. These results extended

our tester-gender findings in the A-803467 experiments (see above), and are consistent with a recent paper showing that pheromones from young men profoundly influence the injured animals, the presence of male pheromones enhances vigilance, including cutaneous sensitivity. Injection of ambroxol (100 mg/kg, i.p.) produced larger reversals of hypersensitivity in SCI animals than it did in shams, and larger reversals than produced by vehicle injections (Figs. 3A, B, C, D). This pattern is encouraging for our hypothesis that Nav1.8 antagonism reduces SCI-induced cutaneous hypersensitivity. However, another complication emerged in these treatment results -- vehicle injection also tended to reverse the cutaneous hypersensitivity. This general effect might represent stress-induced analgesia produced by the needle stick and/or intraperitoneal effects of the vehicle (10% PEG 400 in distilled water). The possibility of stress-induced analgesia is consistent with the larger reversals seen with the male tester, since male pheromone is reported to be analgesic (Sorge et al., 2014).

Given the decreased sensitivity shown in all groups in the treatment test in Fig.3, another potential explanation was that repeated testing by itself caused the hypersensitivity after injury to habituate. Although this had not been observed in our previous studies, possible habituation might be more prominent with a male tester than the female testers used previously. Therefore, we asked how stable

Fig.4



measures of sensitivity to our heat and mechanical stimuli are in naïve (uninjured) animals during repeated stimulation by a male tester (Alexis Bavencoffe). The results in Fig.4 showed the responses on the second test following one earlier habituating test to the same stimuli, which followed a session of habituation to the testing devices. In the experiment summarized in Fig.4, Dr. Bavencoffe delivered a single up-down mechanical stimulus sequence each morning and a heat stimulus sequence each afternoon for 4 consecutive days, following an initial day of habituation to the testing devices. Two different groups of animals were compared (DoDG22 and DoDG23, each with n=8). Importantly, the differences noted between the tests on Day 2 and Day 3, although substantial in some cases, were opposite to what was observed in the corresponding tests in Fig.4. This argues against habituation (if anything, sensitization was found) as an explanation for the treatment effects in Fig.4. In addition, it was interesting that substantial variability occurred both within each group and between the groups. These complex results add to questions being raised by our findings and by growing numbers of other investigators about how useful these tests of reflex sensitivity are as measures of pain, especially given the more promising results being found with operant measures of pain and anxiety (see below).

Task 1e – Investigate behavioral hypersensitivity effects of blocking Nav1.8 activity with repeated low and high doses of ambroxol.

Preliminary studies with multiple intraperitoneal injections of a high dose of ambroxol 6 weeks after SCI failed to show any consistent trends for attenuation of reflex hypersensitivity. The preliminary results in Tasks 1d and 1e, as well as Tasks 2d and 2e, plus concerns about potential side effects of the very high doses of ambroxol used both in our studies and all the published claims of analgesic effects from ambroxol, persuaded us that our remaining effort in this project should focus exclusively on the highly specific Nav1.8 antagonist, A-803467.

Task 1f – Investigate effects on visceral hypersensitivity of selectively blocking Nav1.8 activity with high doses of A-803467.

We reluctantly abandoned this task. In Year 1 we had performed several of these experiments with animals that received Nav1.8 ASO's via intrathecal catheters, with mixed but somewhat encouraging results. However, we realized that response variability and potentially unnecessary suffering was likely from the cumulative stress of SCI followed by catheterization (which interacts adversely with SCI) followed by numerous tests of forelimb and hindlimb reflexes and finally implantation of recording electrodes before noxious visceromotor testing. Therefore, we decided to limit visceromotor testing to the studies using drug application rather than intrathecal ASO application. Before we could continue these studies a critical member of Dr. Hongzhen Hu's laboratory left in Year 1 and then Dr. Hu himself departed unexpectedly at the end of Year 2. Although Dr. Yang trained in the necessary methods, and Dr. Hu left us necessary equipment for conducting these experiments, Dr Yang did not have time to get to these experiments before she left the project at the end of Year 3.

Task 2a – Optimize conditions for the use of operant CPP and OC tests to reveal emotional/cognitive features of SCI pain.

We have continued to work on the most important part of this project - investigating the roles of Nav1.8-dependent nociceptor activity in maintaining the aversive features of pain-related behavior after SCI. In our paper (Yang et al., 2014) we described the first evidence for ongoing, **spontaneous pain** in a contusive SCI model. We modified a conditioned place preference (**CPP**) procedure that had been used to assess ongoing pain in several other rat pain models. An important difference is that we conditioned place preference to a white chamber paired with retigabine injection. In the same animals, vehicle injection was paired with placement in the black chamber in the 3-chambered box (white-gray-black). Retigabine opens KCNQ K^+ channels, reducing neuronal excitability and, in other models, behavioral hypersensitivity. Importantly, we found that retigabine suppresses SA in small DRG neurons and reverses hyperreflexia after SCI (Yang et al., 2014). Another difference is that we habituated the animals to the black and gray chambers before conditioning, but the animals did not experience the white chamber until it was paired with retigabine injection (increasing the salience of the white chamber and its probable effectiveness as a conditioned context). One day after the 3-day differential conditioning procedure, sham animals preferred the vehicle-paired black chamber, whereas SCI animals showed relative preference for the white, retigabine-paired chamber. Preference for the white chamber in SCI but not sham animals indicates that retigabine is only rewarding when an SCI-induced aversive state is present. Using antisense oligodeoxynucleotides to knock down Nav1.8, we showed that Nav1.8 function is necessary to maintain ongoing pain after SCI. We have now confirmed that this CPP procedure is effective in a separately funded study (see also Fig.5), and we taught it to another group (Dr. Annemieke Kavelaars' laboratory at University of Texas MD Anderson Cancer Center), which showed that this new retigabine-based CPP model is effective in another chronic pain model in mice.

Task 2b – Investigate prolonged effects on spontaneous pain of Nav1.8 knockdown

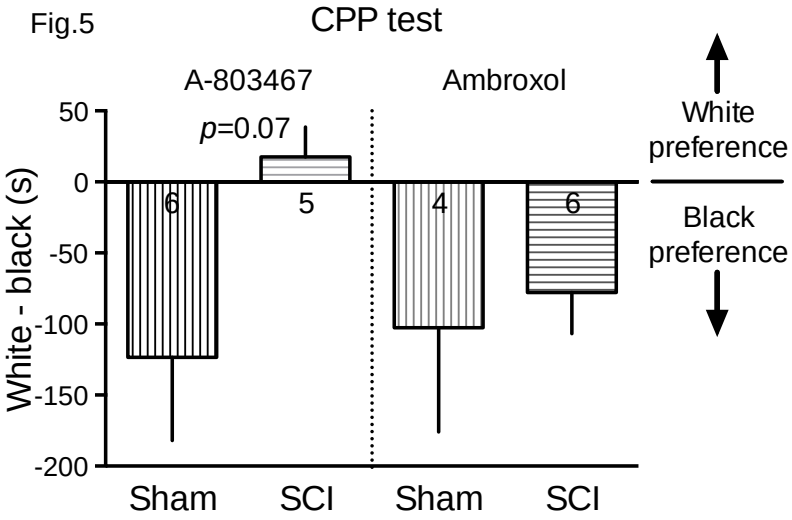
Antisense knockdown of Nav1.8 eliminated signs of spontaneous pain 6-8 weeks after SCI, as assessed with the CPP test. The results are described in our published paper (Yang et al., 2014).

Task 2c – Investigate brief effects on spontaneous pain from a single application of A-803467.

Initial studies conducted by Mr. Odem revealed little or no conditioning of place preference when nociceptor SA was blocked acutely by a single i.p. injection of A-803467 (data not shown). Further experiments using female testers are examined whether the lack of a drug effect represents a failure of brief inhibition of nociceptor SA to provide analgesia, or whether the lack of conditioning is a consequence of complicating stress-induced analgesia produced by the presence of a male tester (Sorge et al., 2014). Interestingly, the successful conditioning of place preference with retigabine (Yang et al., 2014) was achieved when testing was conducted exclusively by a female (Ms. Julia Hadden). Additional tests by a female research volunteer (Alexa van Brummen) yielded promising preliminary evidence that place conditioning in SCI rats occurs when injections of A-803467 but not ambroxol are paired with the innately less-preferred white chamber (vehicle injections are paired with the innately preferred black chamber). In these experiments extensive time was spent by each tester in the presence

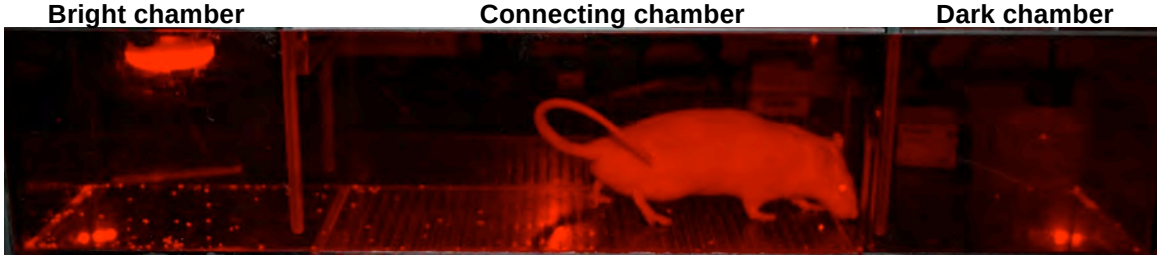
of the rats with frequent handling prior to formal testing so that stressful responses to humans habituated. As shown in Fig. 5 (left panel), the preference showed a strong trend to shift from the vehicle-paired black chamber to the A-803467-paired white chamber. This is an important result for several reasons. First, it provides additional evidence for chronic spontaneous pain in rats after contusive SCI. Second, these results were obtained at the same time and, in part, in the same rats and by the same person (Alexa) who failed to find heat hypersensitivity after contusive SCI in several of the rats that later exhibited CPP. This indicates either that spontaneous pain is a more prominent effect of this type of SCI than heat hypersensitivity, or that some aspect of the procedures (e.g., longer time to dissipate stress from drug injections in the CPP procedure) favors the CPP procedure for revealing pain-related behavior. Consistent with the failure of ambroxol to affect reflex sensitivity (Fig.3), three repeated injections of the much less specific Nav1.8 antagonist, ambroxol, failed to show any evidence for relieving spontaneous pain (Fig.5, right panel).

Fig.5



We have also begun to test the Nav1.8 blocker, A-803467, on an operant measure of **evoked pain**. The Mechanical Conflict System, sold by Coy Labs, has been used in another contusive SCI study (Lau et al., 2012, Neurorehabil Neural Repair, 26, 889-97). The original design of this apparatus was to present a conflict between two aversive stimuli: a very bright light in one chamber and an array of sharp

Fig.6



probes in a connecting chamber that the rat must cross to reach an innately preferred dark chamber (Fig.6). If SCI increases the aversiveness of the probes, the rat can delay crossing the probes or not cross at all. We found 6 or more weeks after our SCI procedure that most rats recover sufficient hindlimb function for full body support (Bedi et al., 2010) and can readily cross the probes, even at their highest level (5 mm). In preliminary studies using a probe height of 3 mm and video analysis we found that compared to the combined control group (4 naïve plus 3 sham rats), SCI rats (n = 8) spent more time in the bright chamber (38 ± 18 vs 8 ± 2 s) before crossing, and when crossing spent less time on the probes (2.5 ± 0.7 vs 5.0 ± 2.0 s), confirming that motor impairment is not a problem. These results, using methods suggested by the developer of the device (Harte et al., 2016) showed considerable variability. We have now modified the procedure to produce a conflict between the animal's strong exploratory drive when introduced to a new environment and aversion to the sharp probes. Thus, instead of going through extensive pre-exposure to the apparatus and probes, we looked at the responses to the first exposure. Rats were placed in the brightly lit chamber for 30 sec, the door to the connecting chamber opened, and the rat allowed to explore, which required it to cross 1 mm high probes. After 5 min the trial was repeated with the probes raised to 4 mm. In a preliminary experiment we used this testing procedure with rats that received intrathecal (i.t.) injection of a small amount of A-803467 or vehicle (counterbalanced order). Preliminary data from 4 SCI rats, 4 sham-operated rats, and 2 naïve rats revealed that all of the sham and naïve rats crossed the probes at least once at both the 1 mm and 4 mm probe heights, regardless of

whether they received vehicle or A-803467 injection 40 min before the test. In contrast, none of the SCI rats crossed at either height when injected with vehicle. Remarkably, half of the SCI rats crossed the probes at both the 1 mm and 4 mm probe heights after i.t. injection of A-803467. These data are incomplete and do not allow any conclusions to be made. However, they are encouraging for our hypothesis that ongoing, Nav1.8-dependent hyperactivity in primary nociceptors is required for hyperalgesia (hypersensitivity to painful stimuli). Furthermore, if continuing experiments replicate this pattern, the i.t. injection site indicates that the locus for nociceptor hyperactivity is not in the neurons' peripheral terminals; it must be in the presynaptic terminals in the spinal cord and/or in the cell bodies within nearby DRGs (which are exposed to CSF and thus to whatever is injected i.t.). These preliminary findings also encourage the use of i.t. injection of Nav1.8 blockers to test the related hypothesis that persistent nociceptor hyperactivity is a major driver of the anxiety that often accompanies SCI pain.

We found strong evidence that **SCI enhances anxiety behavior** in rats, and preliminary evidence that enhanced anxiety may be promoted by activity in Nav1.8-expressing nociceptors. We realized that, when the probes are absent, the 3-chamber Coy box used to test evoked pain is equivalent to a conventional light/dark box used to test anxiety in rodent models. Anxious animals seek shelter and will thus spend more time than unthreatened animals in a dark area when given a choice between a dark area and a brightly lit area where they are more visible (which in nature would increase the risk from predators). Thus, we asked whether SCI animals spend more time than controls (naive and sham) in the dark chamber when placed into the Coy box with the probes completely lowered (0 mm). In each case the rats were placed in the bright chamber. Fig.7 shows that during the first exposure to the unfamiliar Coy box, the SCI rats spent significantly more time in the dark box than did the control. However, by the fourth exposure this preference was gone. Similarly, SCI animals are much less likely to leave the dark chamber once they get there, showing significantly fewer crossings of the connecting chamber during the first exposure but not the fourth exposure to the box (Fig.8). These results suggest that SCI induces anxiety that is expressed as greater preference for the "safer" dark area when the rats find themselves in the unfamiliar box, but that the anxiety is no longer expressed once the SCI rats learn that the context is not threatening.

Fig.7 p = 0.0013 ns

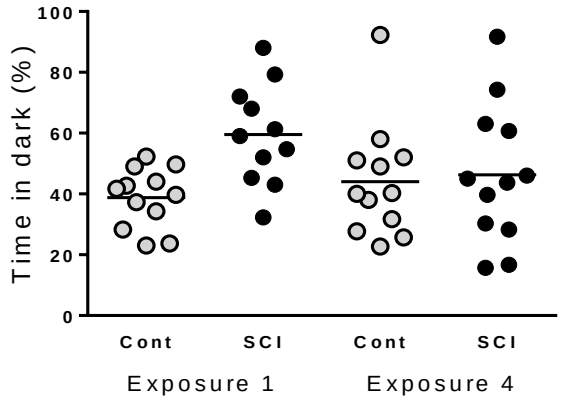
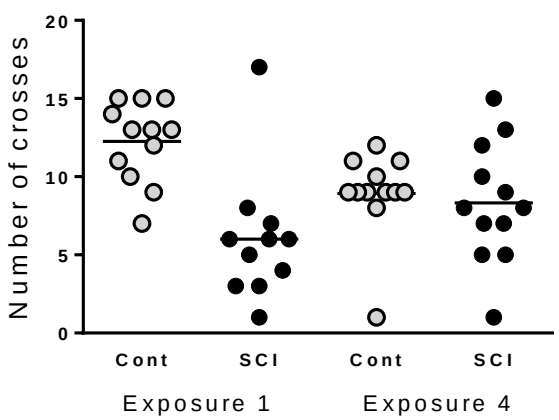


Fig.8 p = 0.0003 ns

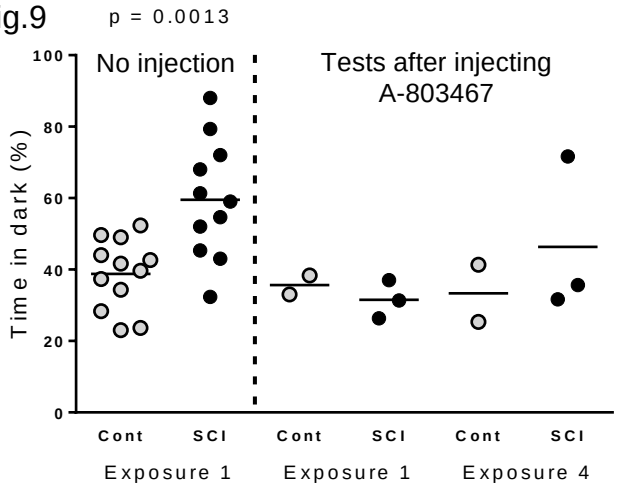


It might be argued that impairment of locomotion explains the lower number of crosses and possibly the greater time spent in the dark by the SCI animals. The increase in number of crosses by the SCI animals during the fourth exposure, to a degree similar to that of the control animals, argues against this possibility, as does the ability of the SCI rats to cross the elevated probes in the later evoked pain phase of these experiments (see above). Additional evidence comes from the similar latencies exhibited by the SCI and control animals to reach the dark chamber after being placed in the white chamber. This was observed during both Exposure 1 (presumably when both groups spend the most time exploring each chamber) and Exposure 4 (when the chambers have become familiar). Taken together, these results indicate that SCI in rats (as has been described in humans) not only produces motivationa/cognitive effects that are expressed as spontaneous and evoked pain, but also produces heightened anxiety that is evident in potentially threatening situations. These results are important for this project because of the

likelihood that SCI-induced pain produces anxiety and that this fully automated operant test for anxiety may serve as a useful indicator of pain-related motivational effects produced by SCI.

Given that the main objective of this award to determine whether motivational/cognitive effects of SCI are driven by hyperactivity in nociceptive primary afferent neurons, it is important to test whether inhibiting hyperactivity in nociceptors by blocking Nav1.8 channels *in vivo* reduces behavioral signs of anxiety. Preliminary experiments support this possibility. The left panel of Fig.6 shows the same data that were presented in Fig.7, which documented the significant preference for the dark by SCI animals during their first exposure to the unfamiliar box. The right panel shows early data suggesting that i.p. injection of A-803467 (100 mg/kg) may eliminate the SCI-induced preference for the dark chamber during the first exposure to the box. If these exciting results are confirmed, they would suggest two equally important possibilities: 1) that chronic nociceptor hyperactivity continuously drives pain, which in turn stimulates other motivationa/cognitive effects including anxiety, and/or 2) that chronic nociceptor hyperactivity can drive anxiety independent of its effects on pain (i.e., a parallel effect rather than an effect in series with pain). Both of these novel possibilities have potentially important clinical implications that would be significant for military personnel.

Fig.9



Task 2d, 2e – Investigate brief effects on spontaneous pain from a single or multiple applications of ambroxol.

Our attempts to produce CPP with 3 injections of ambroxol across 3 days failed to reveal any evidence that ambroxol relieves spontaneous pain.

4. KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrated that intrathecal administration of Nav1.8 antisense oligodeoxynucleotide selectively knocks down Nav1.8 protein expression in lumbar DRGs without significantly reducing the expression of other prominent Na^+ channels in the same ganglia.

2. Discovered that SCI increases the expression of Nav1.8 protein in lumbar DRGs.

3. Confirmed that *in vivo* antisense knockdown of Nav1.8 decreases TTX-resistant inward currents subsequently recorded in dissociated DRG neurons.

4. Demonstrated that antisense knockdown of Nav1.8 reverses SCI-induced reflex hypersensitivity to mechanical and heat test stimuli.

5. Discovered that SCI induces a chronic, aversive state with the properties of spontaneous pain, by showing the conditioning of place preference using a KCNQ channel opener, retigabine, as an analgesic to relieve the pain.

6. Discovered that antisense knockdown of Nav1.8 prevents the conditioning of place preference with retigabine, providing strong evidence that ongoing activity in primary sensory neurons plays a major role in driving chronic spontaneous pain after SCI.

7. Demonstrated that SCI increases Nav1.8 protein in dorsal root ganglia without increasing mRNA expression; i.e., that upregulation occurs by posttranscriptional mechanisms.

8. Confirmed that SCI does not induce Nav1.8 mRNA in the spinal cord.

9. Found complex effects from i.p. injection of specific Nav1.8 antagonist, A-803467, on SCI-induced hypersensitivity to heat and mechanical stimuli. While the antagonist reduced hypersensitivity, its effects were not statistically different from vehicle injection, suggesting possible interfering stress-induced analgesia caused by prolonged injection of the viscous vehicle.

10. Added to evidence that rodent responses to pain-testing stimuli are significantly influenced by the gender of the tester, with male testers eliciting complicating stress responses that are much less apparent when testing is performed by females.

11. Demonstrated that SCI enhances anxiety behavior and obtained preliminary evidence that inhibiting hyperactivity in primary nociceptors may reduce SCI-induced anxiety.

12. Substantially improved SCI surgical procedures and reflex testing methods.

13. Found that high concentrations of the nonspecific Nav1.8 antagonist, ambroxol, fail to reduce SCI-induced hyperreflexia or spontaneous pain.

14. Showed that blockade of a signaling pathway that enhances Nav1.8 activity (the cAMP-PKA pathway) eliminates spontaneous activity in dissociated nociceptors.

5. CONCLUSION

The research accomplishments summarized above provide strong evidence for an important and previously unappreciated role for ongoing hyperactivity in widespread primary afferent neurons for maintaining chronic spontaneous (and possibly evoked) pain, hypersensitivity of defensive reflexes, and possibly anxiety after contusive SCI. Although the complexities of rat behavior and some pharmacological issues (notably, limited bioavailability of A-803467 and lack of efficacy of ambroxol) slowed some aspects of the project, the results suggest that further development of drugs that antagonize Nav1.8 channels could lead to more effective and selective treatments for spontaneous and evoked pain, hyperreflexia (spasticity), and anxiety after SCI. The results also point to a potential role for persistent hyperactivity in widespread nociceptors in other conditions involving chronic pain and anxiety, so the therapeutic implications may generalize beyond SCI. Thus, these discoveries may help to open up new therapeutic approaches to aid many people, including military personnel, suffering from SCI and related painful conditions.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. Manuscripts submitted or published

1. Lay press:

The Spin: Spinal Cord Injury BC, 2015, "Rethinking pain" http://sci-bc.ca/stories/spin-magazine/ Spring 2015.

2. Peer-Reviewed Scientific Journals:

Yang Q, Wu Z, Hadden JK, Odem MA, Zuo Y, Crook RJ, Frost JA, Walters ET (2014) Persistent pain after spinal cord injury is maintained by primary afferent activity. *J Neurosci* 34:10765–10769.

Walters ET (2014) Neuroinflammatory contributions to pain after SCI: roles for central glial mechanisms and nociceptor-mediated host defense. *Exp Neurol* 258:48–61.

(This peer-reviewed, invited review article presented a novel hypothesis that was partly inspired by findings made under Aims 1 and 4 of this project)

Bavencoffe A, Li Y, Wu Z, Yang Q, Herrera J, Kennedy EJ, Walters ET, Dessauer CD. (2016) Persistent electrical activity in primary nociceptors after spinal cord injury is maintained by scaffolded adenylyl cyclase and protein kinase A and is associated with altered adenylyl cyclase regulation. *J Neurosci*. **36**:1660-1668

3. Invited articles:

See Walters (2014) above.

4. Abstracts:

Yang Q, Wu Z, Crook, RJ, Walters ET. Knockdown of Nav1.8 blocks both spontaneous activity in small DRG neurons and reflex hypersensitivity after spinal cord injury. Program No. 67.05. *2012 Abstract Viewer/Itinerary Planner.* New Orleans, LA: Society for Neuroscience, 2012.

Odem MA, Hadden JK, Crook RJ, Du J, Carlton SM, Yang Q, Walters ET. Inhibition of Nav1.8 channels reduces pain-related behavior after spinal cord injury. Program 537.31. *2014 Abstract Viewer/Itinerary Planner.* Washington DC: Society for Neuroscience, 2014.

Bavencoffe A, Wu Z, Yang Q, Du J, Li Y. Kennedy EJ, Carlton SM, Dessauer CW, Walters ET. AKAP-dependent cAMP-PKA signaling maintains pain-related spontaneous activity in nociceptor somata after spinal cord injury. Program 242.26. *2014 Abstract Viewer/Itinerary Planner.* Washington DC: Society for Neuroscience, 2014.

Bavencoffe A, Yang Q, Bloom O, Walters ET. Does macrophage migration inhibitory factor (MIF) contribute to chronic spontaneous activity in nociceptor somata after spinal cord injury? Program 604.09. *2015 Abstract Viewer/Itinerary Planner.* Chicago IL: Society for Neuroscience, 2015.

b. Presentations:

03/06/13 Drexel University, Department of Neurobiology & Anatomy, Philadelphia PA, “Chronic Pain after Spinal Cord Injury is Driven by Widespread Spontaneous Activity in Primary Nociceptors” presented by Edgar T. Walters

05/07/13 Kentucky Spinal Cord and Head Injury Research Symposium, Lexington KY, “Ion Channels in the Cell Bodies of Primary Nociceptors as Potential Targets for Reducing Chronic Pain after SCI” presented by Edgar T. Walters

05/09/13 Symposium “Primary Afferent Hyperexcitability Drives Chronic Pain”, American Pain Society meeting, New Orleans LA, “Spontaneous Activity in Primary Nociceptors May Drive Chronic Pain after Spinal Cord Injury” presented by Edgar T. Walters

09/06/13 International Spinal Research Network Meeting, London UK, “Maladaptive Induction by SCI of an Adaptive Nociceptor State Drives Chronic Neuropathic Pain” presented by Edgar T. Walters

11/22/13 Johns Hopkins Center for Sensory Biology, Baltimore MD, “Primary afferent neurons and TRPV1 channels drive chronic pain after spinal cord injury” presented by Edgar T. Walters

05/07/14 Department of Integrative Biology and Pharmacology, University of Texas Medical School at Houston, " Chronic Pain After Central Injury: Unexpected Mechanisms in Peripheral Sensory Neurons" presented by Edgar T. Walters

10/30/14 Neuroscience Research Seminar Series, Indiana University School of Medicine, Indianapolis, IN, "Hyperactive Primary Nociceptors Drive Chronic Pain After Spinal Cord Injury" presented by Edgar T. Walters

04/16/15 Gulf Coast Consortium for Translational Pain Research Symposium, Houston, TX, "Hyperactive Primary Nociceptors and Chronic Pain after Spinal Cord Injury" presented by Edgar T. Walters

06/10/15 European Pain School, Siena, Italy, "Plasticity in Primary Afferent Neurons that Promotes Central Neuropathic Pain" presented by Edgar T. Walters

08/05/15 Texas Pain Research Consortium Conference, University of Texas at Dallas, TX, "Hyperactive Primary Nociceptors and Chronic Pain after Spinal Cord Injury" presented by Edgar T. Walters

03/01/16 Washington University Department of Anesthesiology, St Louis, MO, “Nociceptor Signaling and Chronic Pain: Evolutionary Clues from Injured Invertebrates and Mammals with Spinal Cord Injury” presented by Edgar T. Walters

7. INVENTIONS, PATENTS AND LICENSES: none

8. REPORTABLE OUTCOMES: none other than the papers and presentations described above

9. OTHER ACHIEVEMENTS: none directly related to this award

10. REFERENCES (***** supported in part by this award)**:**

*Bavencoffe A, Li Y, Wu Z, Yang Q, Herrera J, Kennedy EJ, Walters ET, Dessauer CD. (2016) Persistent electrical activity in primary nociceptors after spinal cord injury is maintained by scaffolded adenylyl cyclase and protein kinase A and is associated with altered adenylyl cyclase regulation. *J Neurosci*. **36**:1660-1668

Bedi, SS, Lago, MT, Masha, LI, Crook, RJ, Grill, RJ, Walters, ET (2012) Spinal cord injury triggers an intrinsic growth-promoting state in nociceptors. J Neurotrauma, 29:925–935.

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Harte, SE, Meyers, JB, Donahue, RR, Taylor, BK, Morrow, TJ (2016) Mechanical Conflict System: A Novel Operant Method for the Assessment of Nociceptive Behavior. PLoS One, 11:e0150164.

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Walters, ET (2012) Nociceptors as chronic drivers of pain and hyperreflexia after spinal cord injury: an adaptive-maladaptive hyperfunctional state hypothesis. Front Physiol, 3:309.

*Walters, ET (2014) Neuroinflammatory contributions to pain after SCI: roles for central glial mechanisms and nociceptor-mediated host defense. Exp Neurol, 258:48–61.

Wu, Z, Yang, Q, Crook, RJ, O'Neil, RG, Walters, ET (2013) TRPV1 channels make major contributions to behavioral hypersensitivity and spontaneous activity in nociceptors after spinal cord injury. Pain, 154:2130–2141.

*Yang, Q, Wu, Z, Hadden, JK, Odem, MA, Zuo, Y, Crook, RJ, Frost, JA, Walters, ET (2014) Persistent pain after spinal cord injury is maintained by primary afferent activity. J Neurosci, 34:10765–10769.

11. APPENDICES

Individuals working on the project

Name	Edgar T. Walters, Ph.D.
Project role	PD/PI
Nearest person month worked	12
Contributions to project	Design, supervision, data analysis, writing, and presentation of results.
Funding support (other than DoD)	National Science Foundation, "Collaborative Research: Comparisons of Fun and Mechanisms of Nociceptive Sensitization in Dissimilar Molluscs". Role - Craig H. Neilsen Foundation, “Contributions of inflammatory mediators in chronic SCI”, Role - collaborator. Mission Connect-TIRR Foundation, "Targeting TRPV1 Channels to Reduce Spontaneous Neuropathic Pain After SCI ". Role - PI.

Name	Qing Yang, M.D.
Project role	Co-PD/PI
Nearest person month worked	12
Contributions to project	Design, electrophysiology, SCI surgery, behavioral tests, animal care, western blot, data analysis, writing.
Funding support (other than DoD)	Mission Connect-TIRR Foundation, "Neuroprotective Effect of Targeting KCNQ/Kv7 Channels in Spinal Cord Injury". Role - PI. American Pain Society, "Novel Target for Preventing & Ameliorating Paclitaxel-Induced Neuropathic Pain". Role - PI.

Name	Alexis Bavencoffe
Project role	Postdoctoral fellow
Nearest person month worked	9
Contributions to project	Electrophysiology, behavioral tests, animal care
Funding support (other than DoD)	

Name	Max Odem
Project role	Research assistant (graduate student)
Nearest person month worked	6
Contributions to project	Design, behavioral tests, animal care, data analysis, SCI surgery
Funding support (other than DoD)	Research assistantship from Graduate School of Biomedical Sciences

Name	Alexa van Brummen
Project role	Research assistant (medical student, summer research)
Nearest person month worked	2
Contributions to project	Behavioral tests, animal care
Funding support (other than DoD)	Endowment to E.T. Walters

Opportunities provided for training and professional development:

The PI continued to offer professional mentoring and career guidance to Dr. Qing Yang (Co-PD/PI and a junior faculty member) and Dr. Alexis Bavencoffe. He also provided extensive guidance and instruction to Mr. Max Odem, a graduate student in the laboratory. Mr. Odem has also benefited from courses and career guidance from the University of Texas at Houston Graduate School of Biomedical Sciences.

Brief Communications

Persistent Pain after Spinal Cord Injury Is Maintained by Primary Afferent Activity

Qing Yang, Zizhen Wu, Julia K. Hadden, Max A. Odem, Yan Zuo, Robyn J. Crook, Jeffrey A. Frost, and Edgar T. Walters
Department of Integrative Biology and Pharmacology, University of Texas Medical School at Houston, Texas, 77030

Chronic pain caused by insults to the CNS (central neuropathic pain) is widely assumed to be maintained exclusively by central mechanisms. However, chronic hyperexcitablility occurs in primary nociceptors after spinal cord injury (SCI), suggesting that SCI pain also depends upon continuing activity of peripheral sensory neurons. The present study in rats (*Rattus norvegicus*) found persistent upregulation after SCI of protein, but not mRNA, for a voltage-gated Na^+ channel, Nav1.8, that is expressed almost exclusively in primary afferent neurons. Selectively knocking down Nav1.8 after SCI suppressed spontaneous activity in dissociated dorsal root ganglion neurons, reversed hypersensitivity of hindlimb withdrawal reflexes, and reduced ongoing pain assessed by a conditioned place preference test. These results show that activity in primary afferent neurons contributes to ongoing SCI pain.

Key words: **Chronic pain; dorsal root ganglion; Nav1.8; neuropathic pain; nociceptor; spinal contusion**

Introduction

After peripheral injury or inflammation, central sensitization in the spinal cord promotes allodynia, hyperalgesia, and spontaneous pain, but this central sensitization often requires continuing sensory activity (Baron et al., 2013). In contrast, pain resulting from injury or inflammation within the CNS (central neuropathic pain) is usually assumed to be maintained by central alterations (Finnerup, 2013). However, recent findings suggest that persistent hyperexcitability and spontaneous activity (SA) in primary sensory neurons might promote spinal cord injury (SCI) pain. First, SA occurs in peripheral terminals (Carlton et al., 2009) and in cell bodies of nociceptors *in vivo* and after dissociation from dorsal root ganglia (DRG; Bedi et al., 2010) long after SCI. Second, reduction of TRPV1 function reverses behavioral hypersensitivity after SCI (Wu et al., 2013). TRPV1 is expressed most abundantly in primary nociceptors (Cavanaugh et al., 2011), suggesting that activity in primary sensory neurons might drive reflex hypersensitivity after SCI. Furthermore, the aversive quality of pain (Baastrup et al., 2010; Navratilova et al., 2013) might also be driven by primary afferent activity after SCI.

A strong test of the hypothesis that activity in primary afferent neurons helps to maintain SCI pain (Walters, 2012) is enabled by the selective expression of a voltage-gated Na^+ channel, Nav1.8, in somatic sensory neurons. Nav1.8 is absent in central neurons (Akopian et al., 1999; Shields et al., 2012) and is important for SA in primary afferent neurons after peripheral insults (Roza et al., 2003; Jarvis et al., 2007). Here, we report that knockdown of Nav1.8 channels after SCI reduces SA in primary afferent neurons, reverses reflex hypersensitivity, and ameliorates a pain-like aversive state.

Materials and Methods

Procedures. All procedures complied with guidelines of the International Association for the Study of Pain and were approved by the institutional animal care and use committee. Male rats (200–350 g) were maintained under a 12 h reversed light/dark cycle and tested during the dark phase. Additional methodological details are available (Bedi et al., 2010; Wu et al., 2013).

SCI procedures. Contusion injury occurred at vertebral segment T10 (Bedi et al., 2010). Rats were deeply anesthetized with ketamine (80 mg/kg), xylazine (10 mg/kg), and acepromazine (0.75 mg/kg) before laminectomy at T10, followed by a spinal impact using an Infinite Horizon impactor (150 kdyne, 1 s dwell time). Sham-operated ("sham") animals received identical procedures except for spinal impact. Animals accepted for study exhibited Basso, Beattie, and Bresnahan (BBB) hindlimb motor scores of 0 1 d after SCI (Basso et al., 1995). All showed partial locomotor recovery by the end of testing, with extensive movement of all joints in the hindlimbs (BBB score $\geq$7).

Antisense oligodeoxynucleotide (ODN) knockdown of Nav1.8. Previous studies identified an antisense oligodeoxynucleotide (ASO) sequence that is taken up *in vivo* by DRG neurons after intrathecal delivery and reduces expression of Nav1.8 protein (Porreca et al., 1999; Lai et al., 2002). This sequence, 5′-TCC-TCT-GTG-CTT-GGT-TCT-GGC-CT-3′, and a mismatched oligodeoxynucleotide (MMO) sequence, 5′-TCC-TTC-GTG-CTG-TGT-TCG-TGC-CT-3′, were purchased from Sigma-Aldrich. Approximately 1 month after SCI, rats were anesthetized with isoflurane and a chronic intrathecal catheter was inserted at the atlantooccipital joint terminating at the lumbar enlargement. Animals showing additional impairment after catheterization (altered body posture or forelimb function) were killed. Intrathecal injections (45 μg of ODN in 5 μl of saline, followed by a 10 μl of saline flush) were given 1–2 months after injury, twice daily for 3 d, and then once daily for 2 d.

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Author contributions: Q.Y., Z.W., R.J.C., J.A.F., and E.T.W. designed research; Q.Y., Z.W., J.K.H., M.A.O., Y.Z., and R.J.C. performed research; Q.Y., Z.W., M.A.O., Y.Z., R.J.C., J.A.F., and E.T.W. analyzed data; E.T.W. wrote the paper.

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The authors declare no competing financial interests.

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Western blot analysis of Nav protein expression. At the end of ODN injection, animals were deeply anesthetized and bilateral L4 and L5 ganglia were removed and immediately homogenized in RIPA buffer (Teknova) with protease inhibitors. Lysate protein concentrations were determined by bicinchoninic acid assay (Pierce). Equal amounts of total protein (30 μg) were resolved by SDS-PAGE (4–20% Tris-HCl; Bio-Rad) after 1:1 dilution with Laemmli buffer, transferred to a PVDF membrane, blocked with 10% nonfat dry milk, and incubated overnight at 4°C with antibodies against Nav1.8 (catalog #AB9274; Millipore), Nav1.6 (catalog #ASC-009; Alomone Labs), Nav1.7 (catalog #ASC-008; Alomone Labs), or Nav1.9 (catalog #AB-9222; Millipore), and β-actin (catalog #ab6276; Abcam). Secondary HRP anti-rabbit or anti-mouse IgGs were incubated for 1 h at 22°C. Blots were developed using an enhanced chemiluminescence substrate (Pierce). Optical densities were normalized to β-actin.

RT-PCR analysis of Nav1.8 mRNA expression in DRG neurons and spinal cord. Total RNA was extracted from homogenized DRG or spinal cord with on-column DNase digestion (E.Z.N.A. Total RNA Kit I) and cDNA was synthesized by MMLV reverse transcriptase (Invitrogen) using random primer. Rat Nav1.8 primers were TCCCGGGGAAGGCTACATTA (forward) and TAATGTTGGCCCGGTCACTC (reverse; Hu et al., 2013); rat GAPDH primers were CCCCCAATGTATCCGTTGTG (forward) and TAGCCCAGGATGCCCTTTAGT (reverse; Piller et al., 2013). mRNA abundance was determined by real-time PCR (LightCycler 480; Roche) with SYBR Green Master Mix (Sigma). Preincubation at 95°C for 3 min was followed by 45 amplification cycles (95°C for 30 s, 57°C for 30 s, and 72°C for 30 s) and fluorescence collection at 60°C. Gene expression was normalized to *Gapdh* and expressed as fold change of sham control averaged over three replicates from each of three animals in each group.

Dissociation and culture of DRG neurons. Selected DRG neurons (L5, L4, T12, T11, T9, T8) were minced and incubated for 40 min at 34°C with trypsin (0.4 mg/ml) and collagenase D (1.6 mg/ml). DRG fragments were triturated and the neurons were plated without serum or growth factors onto dishes coated with poly-L-lysine and kept overnight in DMEM under 5% CO_2, 95% humidity at 37°C.

Recording from dissociated DRG neurons. Whole-cell patch recordings of SA were made at ~23°C from small neurons 18–26 h after dissociation, as described previously (Bedi et al., 2010). Tetrodotoxin (TTX)-resistant currents were measured in solution containing the following (in mM): 130 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 0.1 $CdCl_2$, 10 TEA-Cl, 10 HEPES, and 5 glucose, and a pipette solution containing the following (in mM): 100 CsCl, 30 CsF, 8 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 0.4 Na2GTP, 4 MgATP, 10 EGTA, and 10 HEPES. Current–voltage relationships were determined with 250 nM TTX and a holding potential of −60 mV to isolate Nav1.8 channels (Cummins et al., 1999); 200 ms command potentials were delivered from −100 to 50 mV in 10 mV increments at 5 s intervals, with each command following a 100 ms conditioning prepulse to −120 mV.

Reflex hypersensitivity tests. Tests were conducted by investigators blinded to treatment before injury and just before and at the end of ODN treatment (Bedi et al., 2010; Wu et al., 2013). Animals were habituated to test chambers on day 1. On days 2 and 3, heat and mechanical test stimuli were given for habituation to test procedures; data were collected from tests on days 4 and 5. Hindlimb heat hypersensitivity was tested by the Hargreaves radiant heat method. Mechanical hypersensitivity was tested with calibrated von Frey filaments delivered to the glabrous surface of hindpaws.

Conditioned place preference test for ongoing pain. We modified conditioned place preference (CPP) procedures used in peripheral pain models (King et al., 2009). A commercial CPP box (Med Associates) with automated data collection had three chambers with equal levels of dim illumination: black and white end chambers and a connecting gray chamber. On day 1 (~2 months after injury), each rat was permitted to explore the gray and black (but not white) chambers. Conditioning trials occurred on days 2–4. Each morning, 0.5 ml of vehicle (saline) was injected intraperitoneally 10 min before confinement in the black chamber for 60 min. Three hours later, the same rat was injected with retigabine (10 mg/kg in 0.5 ml of saline; Yang et al., 2013) 10 min before confinement in the white chamber. On day 5, each rat was placed in the gray chamber with unrestricted access to all chambers for 15 min. Data are presented as the difference in time spent in the retigabine-paired (white) chamber minus time in the vehicle-paired (black) chamber during the drug-free day 5 test. The total number of crossings into all three chambers provided objective criteria to exclude SCI animals exhibiting excessive locomotor impairment (<21 crossings; two SCI + MMO and two SCI + ASO animals) or insufficient spinal injury (>250 crossings; one SCI + MMO and one SCI + ASO animal). For comparison, sham animals exhibited 190 ± 26 total crossings during the test.

Statistical analysis. Data are presented as means ± SEM. Comparisons were made with Student's *t* tests or one- or two-way ANOVA, followed by Bonferroni's *post hoc* tests. SA incidence was compared using Fisher's exact tests.

Results

Nav1.8 antisense treatment selectively reduces Nav1.8 expression in DRG neurons after upregulation by SCI

We investigated whether SCI had any effect on Nav1.8 expression in L4 and L5 DRG. These DRG are sufficiently far from the vertebral T10 contusion site that few C-fiber neurons should be injured directly by the T10 injury (Bedi et al., 2010), but neurons in these DRG may be exposed to inflammatory signals disseminated after SCI (McKay and McLachlan, 2004; Alexander and Popovich, 2009), which might alter Nav1.8 expression (Yu et al., 2011). Processing for Western blot analysis began immediately after excision of L4 and L5 DRG 1 month after injury (Fig. 1*A*). The amount of Nav1.8 protein differed among DRG from naive, sham, and SCI animals ($F_{(2,11)} = 8.83$; $p = 0.005$), with the levels being significantly higher in ganglia from SCI than naive or sham animals ($p < 0.01$ and $p < 0.05$, respectively). No difference was found between SCI and sham groups in Nav1.8 mRNA expression in lumbar DRG, nor was any evidence found for Nav1.8 mRNA expression in lumbar spinal cord in either group (Fig. 1*B*). Using a Nav1.8 ASO sequence (Lai et al., 2002), we compared the expression of four different Nav proteins after Nav1.8 ASO treatment to the corresponding expression after Nav1.8 MMO treatment (Fig. 1*C*). No significant differences were found in expression of Nav1.6, Nav1.7, or Nav1.9 channels in SCI animals after lumbar Nav1.8 ASO treatment, whereas Nav1.8 protein expression was significantly lower after Nav1.8 ASO treatment than after MMO treatment ($p = 0.002$).

Nav1.8 antisense treatment reduces TTX-resistant Na^+ current and spontaneous activity in DRG neurons after SCI

Nav1.8 channels mediate a distinctive Na^+ current that is resistant to TTX and has an unusually depolarized voltage dependence of fast inactivation (Akopian et al., 1996; Dib-Hajj et al., 1997). We assessed the degree of reduction of Nav1.8-mediated, TTX-resistant Na^+ current by antisense knockdown in small DRG neurons from SCI animals. In the presence of 250 nM TTX, a whole-cell voltage-clamp pulse protocol that inactivates the other TTX-resistant Nav channel in DRG neurons, Nav1.9, was used (Cummins et al., 1999). Depolarizing steps from a −60 mV holding potential produced inward currents with relatively slow activation and inactivation kinetics (Fig. 2*A*, left), with a threshold level of ~−30 mV and a peak at ~0 mV (Fig. 2*A*, middle), suggestive of an Nav1.8-mediated current. The peak amplitude of evoked current was lower in DRG neurons after *in vivo* Nav1.8 ASO injections (Fig. 2*A*, right) than after MMO injections when tested ~20 h ($p < 0.0001$, unpaired *t* test), but not ~40 h, after the last injection, consistent with known rates of turnover of Nav channels (Porreca et al., 1999).

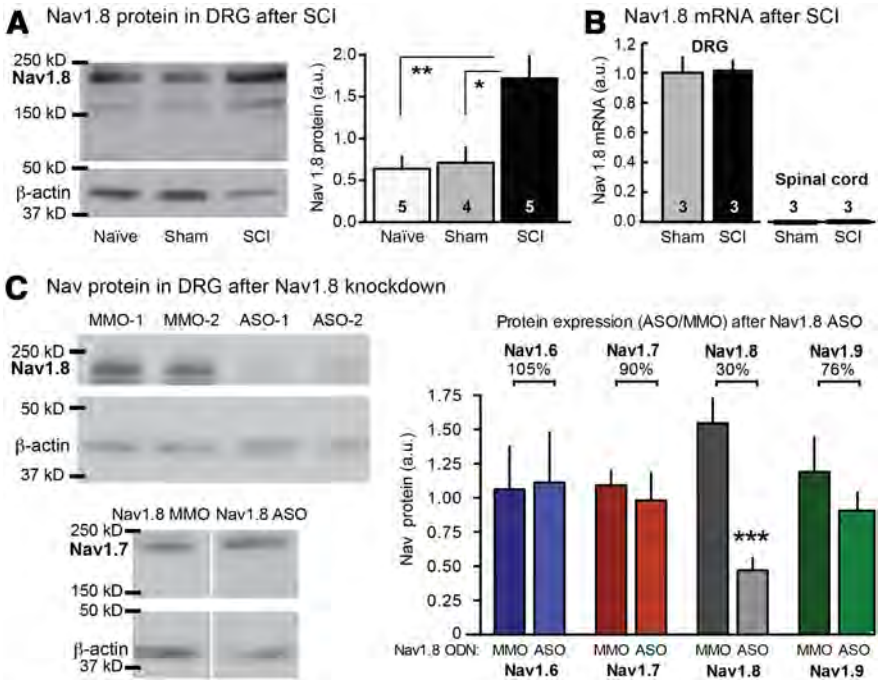

Figure 1. Effects of SCI and Nav1.8 antisense treatment on Nav channel expression. ***A***, SCI increases Nav1.8 protein in L4 and L5 DRG 1 month after injury. Left, Sample Western blots. Right, Increase in Nav1.8 protein after SCI (normalized to β-actin). Number of animals tested (four DRG per animal) is indicated in each bar. ***B***, Nav1.8 mRNA expression is detectable in DRG, but not spinal cord, and is not altered by SCI. ***C***, Nav1.8 ASO treatment selectively knocks down Nav1.8 protein expression. Left, Sample Western blot (two animals each for Nav1.8 and one each for Nav1.7). Right, Normalized expression of four Nav channels. Percentages indicate mean relative expression for each channel protein after Nav1.8 ASO treatment compared with Nav1.8 MMO treatment.

An important prediction was that knockdown of Nav1.8 expression would reduce SCI-induced SA. SCI quadrupled the incidence of SA in small DRG neurons (Fig. 2*B*, left) dissociated from L4 and L5 ganglia 3 d after injury compared with neurons dissociated from naive animals ($p < 0.0001$; Fig. 2*B*, right), and this increase was abolished by Nav1.8 ASO injections at the lumbar level ($p = 0.003$ vs SCI). Similarly, SCI increased the incidence of SA 1–3 months after SCI, and this increase was abolished by lumbar SCI + ASO treatment ($p = 0.006$). SA incidence was significantly greater in lumbar DRG neurons after SCI or SCI + MMO treatment than in lumbar DRG neurons after lumbar SCI + ASO treatment ($p = 0.003$). No significant effects on SCI-induced increases in SA were found in DRG neurons dissociated from thoracic ganglia (Fig. 2*B*, right) after Nav1.8 ASO treatment at the lumbar level.

Nav1.8 antisense treatment reverses hindlimb hyperreflexia and ongoing pain after SCI

Persistent sensitization of hindlimb withdrawal responses evoked by mechanical and heat test stimuli was found 1–3 months after SCI, similar to earlier comparisons of SCI, sham, and naive groups (Bedi et al., 2010), and this sensitization was reversed by Nav1.8 ASO treatment. For mechanical sensitivity (Fig. 3*A*), significant effects were found for test sequence (pre-SCI, post-SCI, post-ODN; $F_{(2,84)} = 23.9$; $p < 0.0001$), ODN treatment ($F_{(1,57)} = 4.5$; $p = 0.039$), and their interaction ($F_{(2,57)} = 4.82$; $p = 0.0012$). *Post hoc* comparison revealed higher post-ODN withdrawal threshold in ASO-treated than MMO-treated rats ($p = 0.0009$). Twenty-eight of 29 animals showed mechanical hypersensitivity after SCI (before ODN treatment) and 72% also showed heat hypersensitivity. In these animals, significant effects were found for test sequence ($F_{(2,57)} = 14.2$; $p < 0.0001$), ODN treatment ($F_{(1,9)} = 11.03$; $p = 0.009$), and their interaction ($F_{(2,57)} = 4.82$; $p = 0.011$). *Post hoc* comparison revealed higher post-ODN withdrawal latency in ASO-treated than in MMO-treated rats ($p = 0.0019$).

To investigate ongoing pain, we used operant conditioning of place preference to a chamber paired with retigabine injection. Retigabine opens KCNQ K^+ channels, reducing neuronal excitability and, in other models, behavioral hypersensitivity (Brown and Passmore, 2009). Importantly, we found recently that retigabine suppresses SA in small DRG neurons and reverses hyperreflexia after SCI (Yang et al., 2013). One day after the 3 d differential conditioning procedure, sham animals preferred the vehicle-paired black chamber (as do naive animals, unpublished observations), whereas SCI animals showed relative preference for the white, retigabine-paired chamber ($p = 0.003$, unpaired *t* test; Fig. 3*C*, left). Preference for the white chamber in SCI, but not sham, animals indicates that retigabine is only rewarding when an SCI-induced aversive state is present. To test whether knockdown of Nav1.8 reduces the conditioned shift in preference toward the white chamber in SCI animals, we tested animals after MMO or ASO treatment and found that, compared with the SCI + MMO animals, SCI + ASO animals significantly preferred the black chamber ($p = 0.045$; Fig. 3*C*, right). The absence in SCI + ASO animals of a shift in preference away from the innately preferred, vehicle-paired black chamber suggests that Nav1.8 function is necessary to maintain ongoing pain after SCI.

Discussion

Finding that persistent pain induced by SCI requires Nav1.8, which is expressed almost exclusively in primary afferent neurons (Akopian et al., 1999; Shields et al., 2012), indicates that Nav1.8-expressing sensory neurons play a major role in driving SCI pain and perhaps associated central neuropathic alterations (Finnerup, 2013). Our results confirm that intrathecal application of a Nav1.8 ASO sequence reduces expression of Nav1.8 protein in DRG and functional activity of Nav1.8 channels (Porreca et al., 1999; Lai et al., 2002; Gold et al., 2003; Yu et al., 2011). We also demonstrate that this knockdown is highly specific; Nav1.8 ASO treatment did not significantly reduce DRG expression of related Na^+ channels: Nav1.6 and Nav1. 7 (see also Porreca et al., 1999) and Nav 1.9. Reversal of reflex hypersensitivity by Nav1.8 ASO treatment has also been shown in peripheral injury and inflammation models (Yoshimura et al., 2001; Villarreal et al., 2005; Joshi et al., 2006; Morgan and Gebhart, 2008; Miao et al., 2010).

Our hypothesis that SA in primary afferent neurons drives chronic pain after SCI (Bedi et al., 2010; Walters, 2012) is supported by three findings. First, Nav1.8 mRNA was expressed in DRG, but not the spinal cord, even after SCI. Second, Nav1.8 knockdown eliminated the increase in SA in DRG neurons and hyperreflexia after SCI, suggesting that electrical activity in DRG neurons maintains hyperreflexia. This probably explains the strong correlation between the incidence of SA in dissociated

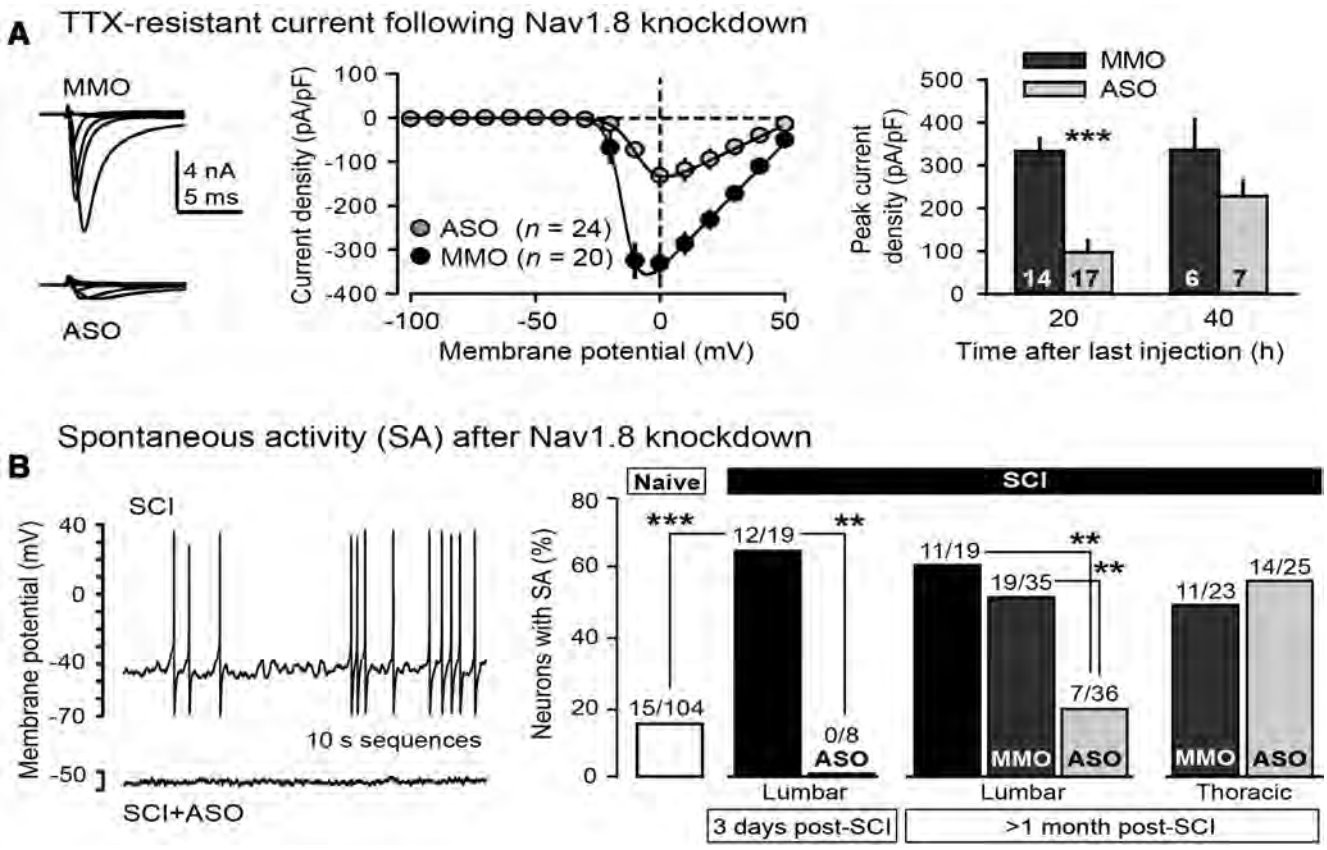

Figure 2. *In vivo* Nav1.8 antisense treatment reduces TTX-resistant Na^+ current and SA in small primary afferent neurons tested *in vitro* after SCI. ***A***, Reduction of TTX-resistant current 18–24 h after Nav1.8 ASO injection. Depolarizing steps evoked smaller currents compared with MMO treatment (left and middle). Peak TTX-resistant current was significantly reduced ~20 h, but not ~40 h, after the last ASO injection (right). ***B***, ASO treatment decreased the incidence of SA (example in left) 3 d and 1–2 months after SCI (right). Significant suppression from lumbar Nav1.8 ASO injection occurred in lumbar, but not thoracic (T8, T9, T11, T12), DRG neurons.

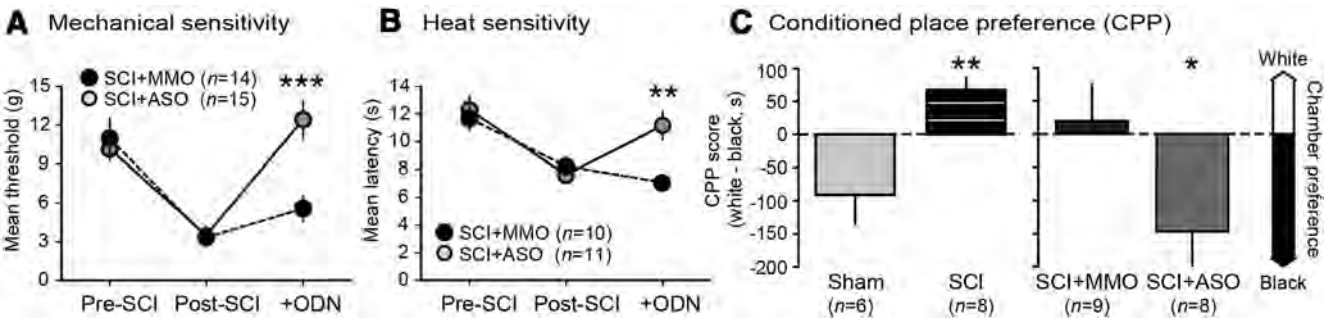

Figure 3. *In vivo* Nav1.8 ASO treatment reverses behavioral indications of hyperreflexia and pain 1–3 months after SCI. ***A***, Nav1.8 ASO treatment reversed the reduction in mechanical threshold for hindlimb withdrawal. ***B***, Nav1.8 ASO treatment reversed the reduction in latency for withdrawal to heat. ***C***, Intraperitoneal injections of retigabine supported CPP after SCI (left), which was prevented in SCI animals by Nav1.8 ASO treatment (right). CPP score (left axis) quantifies relative preference (indicated by arrows on right) for the white (retigabine paired) and black (vehicle paired) chambers after conditioning.

DRG neurons and the degree of reflex hypersensitivity (Bedi et al., 2010). Third, an intervention that selectively reduces activity in primary sensory neurons (Nav1.8 knockdown) blocks a CPP measure of ongoing pain that captures its aversive quality (Navratilova et al., 2013). Because retigabine's effects may involve central as well as peripheral mechanisms (Brown and Passmore, 2009), it was important that the drug was only present during conditioning, not testing. Suppression by retigabine of ongoing pain during conditioning rather than long-lasting central actions or intrinsic reward value was indicated by the lack of retigabine-dependent CPP in sham, naive (unpublished observations), or Nav1.8 SCI + ASO animals. The finding of retigabine-dependent CPP after SCI adds to operant evidence for SCI-induced aversive states in rodents (Baastrup et al., 2010; Davoody et al., 2011; Lau et al., 2012; Vierck et al., 2013).

In many nociceptors, Nav1.8 channels are responsible for the upstroke of the action potential (Renganathan et al., 2001), which could explain suppression of SA by knockdown of Nav1.8. Upregulation of Nav1.8 after SCI is likely to contribute to generalized nociceptor hyperexcitability and might contribute to increased SA (Choi and Waxman, 2011), although multiple electrophysiological alterations probably promote nociceptor SA after SCI (Bedi et al., 2010; Wu et al., 2013). Regardless of the complexity of SA mechanisms, the requirement for Nav1.8 sug-

gests that interventions preferentially targeting nociceptive primary afferent neurons, such as antagonists of Nav1.8 (Jarvis et al., 2007) or TRPV1 (Wu et al., 2013), may relieve some forms of central neuropathic pain.

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Review

Neuroinflammatory contributions to pain after SCI: Roles for central glial mechanisms and nociceptor-mediated host defense

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ABSTRACT

Neuropathic pain after spinal cord injury (SCI) is common, often intractable, and can be severely debilitating. A number of mechanisms have been proposed for this pain, which are discussed briefly, along with methods for revealing SCI pain in animal models, such as the recently applied conditioned place preference test. During the last decade, studies of animal models have shown that both central neuroinflammation and behavioral hypersensitivity (indirect reflex measures of pain) persist chronically after SCI. Interventions that reduce neuroinflammation have been found to ameliorate pain-related behavior, such as treatment with agents that inhibit the activation states of microglia and/or astroglia (including IL-10, minocycline, etanercept, propentofylline, ibudilast, licofelone, SP600125, carbenoxolone). Reversal of pain-related behavior has also been shown with disruption by an inhibitor (CR8) and/or genetic deletion of cell cycle-related proteins, deletion of a truncated receptor (trkB.T1) for brain-derived neurotrophic factor (BDNF), or reduction by antisense knockdown or an inhibitor (AMG9810) of the activity of channels (TRPV1 or Nav1.8) important for electrical activity in primary nociceptors. Nociceptor activity is known to drive central neuroinflammation in peripheral injury models, and nociceptors appear to be an integral component of host defense. Thus, emerging results suggest that spinal and systemic effects of SCI can activate nociceptor-mediated host defense responses that interact via neuroinflammatory signaling with complex central consequences of SCI to drive chronic pain. This broader view of SCI-induced neuroinflammation suggests new targets, and additional complications, for efforts to develop effective treatments for neuropathic SCI pain.

Contents

Introduction

While our everyday experiences with acute inflammation highlight the close association between inflammation and pain, it is only within the last decade that investigators have linked neuroinflammatory consequences of SCI to the life-long, intractable pain that many SCI patients endure. Neuroinflammation refers to the inflammatory responses of the nervous system to pathogens, trauma, toxins, or neurodegeneration. The realization that widespread neuroinflammation plays a major role in driving neuropathic pain after SCI has been accompanied by

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discoveries of unexpected overlap of neuroinflammatory mechanisms that drive persistent pain after both injuries to the central nervous system and to peripheral tissues. This review discusses experimental evidence and a new conceptual perspective that provide insight into how neuroinflammation contributes to SCI pain. Although progress in developing effective treatments for neuropathic SCI pain has been slow, a number of recent preclinical findings suggest novel therapeutic targets that may offer promise as additional treatment options.

Prevalence, types, and properties of pain after SCI

Estimates of its prevalence vary greatly, but it is likely that chronic pain afflicts a majority of SCI patients (Dijkers et al., 2009). This pain is typically divided into two major classes, nociceptive and neuropathic (Bryce et al., 2012). The largest prospective pain study of patients with traumatic SCI found that moderate-to-severe nociceptive and neuropathic pain was equally common (each affecting 59% of patients) one year after SCI (Finnerup et al., 2014). A third class, "other pain," is much less common after SCI (Finnerup et al., 2014) and won't be considered here. *Nociceptive pain* is caused by an activity generated by normal mechanisms in the peripheral terminals of primary nociceptors. This class of pain is a natural response to common sequelae of SCI (Finnerup and Baastrup, 2012), including overuse of the upper body, muscle weakness, poor posture, spasticity, and other problems of the musculoskeletal system. Nociceptive visceral pain after SCI can be caused by constipation, nociceptive cutaneous pain from pressure sores, and nociceptive headache from autonomic dysreflexia. Some forms of nociceptive pain are likely to involve peripheral inflammation, including peripheral neurogenic inflammation promoted by a release into the peripheral tissues of peptides such as calcitonin gene-related peptide (CGRP) and substance P from terminals of primary nociceptors (Richardson and Vasko, 2002; Xanthos and Sandkuhler, 2014).

Neuropathic pain is defined as resulting directly from damage to or disease of the nervous system (Jensen et al., 2011). Neuropathic pain caused by SCI, like other forms of neuropathic pain (Costigan et al., 2009), is generally considered a purely pathological, maladaptive consequence of damage to the nervous system (Gwak and Hulsebosch, 2011; Walters, 2012). Neuropathic pain after SCI occurs in 40–50% of patients, it is often permanent and intractable to available treatments, and it can be one of the most debilitating results of SCI (Finnerup and Baastrup, 2012). Neuropathic SCI pain is subdivided into at-level and below-level pain, felt "at" the spinal injury level (defined as bodily pain felt at the injury level and up to 3 segments rostral to the level) and below the injury level (Bryce et al., 2012). Both types of neuropathic pain summon descriptors such as hot-burning, sharp, shooting, electric shock-like, tingling, squeezing, painfully cold, pricking, and/or pins and needles. This pain often occurs spontaneously and can also be evoked by stimuli that either are not normally painful (allodynia), or in exaggerated form by noxious stimuli (hyperalgesia). Because of its frequent severity and resistance to treatment, neuropathic pain has received far more experimental study than has nociceptive pain after SCI. However, as will be discussed below, persisting nociceptive pain and chronic neuropathic pain after SCI may have partially overlapping neuroinflammatory mechanisms.

The use of animal models to study mechanisms of neuropathic SCI pain

As mentioned in other articles in this special issue, SCI is always followed by neuroinflammation. Clinical observations, including chronic elevation of proinflammatory cytokines in the CSF and blood of patients (Davies et al., 2007; Hayes et al., 2002; Kwon et al., 2010; Segal et al., 1997; Stein et al., 2013), are consistent with the possibility that neuroinflammation that may promote pain persists long after SCI, but direct mechanistic studies in patients have not been performed. The most compelling evidence for neuroinflammatory contributions to neuropathic pain after SCI has come from animal studies, primarily in rodents. The general strategy in these studies has been to explore the behavioral and cellular effects of an SCI produced by one of several, relatively standard procedures, usually applied to a thoracic segment but sometimes to a cervical or upper lumbar segment. These include contusion caused by an impact on the exposed dural surface of the cord, brief compression of the cord by a clip, surgical hemisection, discrete lesion (often electrolytic) of the anterolateral tract, localized excitotoxicity produced by injection of a glutamate receptor agonist, or dorsal root avulsion that damages the dorsal horn (Christensen et al., 1996; Detloff et al., 2013; Hulsebosch et al., 2009; Onifer et al., 2007; Siddall et al., 1995; Vierck et al., 2000; Wieseler et al., 2010; Yezierski et al., 1998; Young, 2002). The most common index of pain in animal studies has been an increase in cutaneous mechanical sensitivity measured as a decrease in the bending force required to elicit a behavioral response by a series of "von Frey" filaments of different stiffness, usually applied to the plantar surface of a hindpaw. Another common index of pain is an increase in heat sensitivity measured as a decrease in latency to withdraw to a radiant stimulus applied to the same site (Hargreaves test).

A limitation of commonly used tests of "pain" after SCI is that the responses monitored are spinally mediated reflexes that may reveal very little about crucial emotional and cognitive aspects of pain, which appear to be cortically mediated (e.g., Mendell, 2011; Wiech et al., 2008). This problem is especially serious when below-level reflex measures are used, such as the pervasive hindlimb "mechanical allodynia" and "thermal hyperalgesia" tests performed with von Frey hairs and radiant heat, because even moderately severe SCI may cause substantial interruption of ascending tracts (Baastrup et al., 2010; Detloff et al., 2008). Behaviors requiring supraspinal processing, such as vocalization, licking, guarding, and facial grimacing, may be more closely associated with affective SCI pain (Baastrup et al., 2010; Bedi et al., 2010; Crown et al., 2008; Sotocinal et al., 2011; Yezierski and Vierck, 2010). Operant tests, where an animal can choose to avoid or escape apparent pain, are being used increasingly as measures of the effects of putative analgesics on the affective, aversive dimension of pain (Ewan and Martin, 2013; Navratilova et al., 2013), including pain after SCI (Lau et al., 2012; Vierck et al., 2013).

One of the most promising indices of pain-like emotional states is offered by the conditioned place preference (CPP) test, an operant measure that clearly reveals the cognitively accessible aversiveness of ongoing pain-like states in animals (Navratilova et al., 2013). The CPP test for pain was first applied to peripheral inflammatory and neuropathic pain models (King et al., 2009; Okun et al., 2011; Sufka, 1994) and showed that a hurt rat will choose to spend more time in a chamber in which it had earlier received relief from pain by injection of an effective analgesic than in a chamber associated with injection of vehicle. Advantages of this test include 1) it can be completely automated, removing the variability in hand-delivered stimulation that complicates standard reflex measures of "pain", 2) the analgesic drug is only present during the conditioning phase after SCI, not during testing a day or more later, so it avoids potentially confounding motor and sensory effects of the drug on behavior during the test, and 3) because the test depends critically on motivationally driven learning after SCI, it reveals higher-order aversive and cognitive aspects of pain rather than lower-order sensory and motor alterations produced by SCI. Limitations of the CPP test in revealing ongoing SCI pain are that 1) the conditioning treatment must relieve pain without being intrinsically rewarding in the absence of pain (e.g., opioids cannot be used) and 2) it is necessary that injured animals be able to move readily from non-preferred chambers to a preferred chamber during the test, which is more difficult in partially paralyzed animals after SCI. However, locomotion eventually recovers sufficiently in most rodent SCI models to permit ready movement between test chambers if the test trial is long enough. Two recent studies have used the CPP test to reveal an ongoing pain-like state in rats after SCI. Rats that received an electrolytic lesion in the ventrolateral

quadrant of the cervical cord showed not only long-lasting mechanical hypersensitivity of hindlimb reflexes, but preferred a chamber that had been paired a month after SCI with an analgesic treatment: either intraventricular injection of an alpha adrenergic agonist, clonidine, or electrical stimulation of motor cortex (Davoody et al., 2011). The lack of preference when these treatments were given to sham-treated rats showed that they were not intrinsically rewarding, but instead depended on the relief of an aversive state caused by SCI. In addition, significant CPP has been found in rats 6 to 8 weeks after thoracic contusion injury using a drug, retigabine, that reduces mechanical and heat hypersensitivity after SCI but has no effect on place preference in naïve or sham-treated animals lacking CNS injury (Yang, Q., Hadden, J., Crook, R.J., Walters, E.T., unpublished observations). It will be important to see if CPP is also produced by interventions that explicitly target neuroinflammatory mechanisms proposed to drive pain.

Types of mechanisms implicated in neuropathic SCI pain

Neuroinflammatory mechanisms are just one set of mechanisms driving SCI pain, and will be reviewed in the following sections. Other general mechanisms have also been proposed, and these may operate independently or synergistically with neuroinflammation to promote SCI pain. Some of these represent direct effects of injury on spinal tissue. An immediate effect of spinal injury is intense activation of nearby neurons by depolarizing agents released during the injury (e.g., K^+ and glutamate from dying and injured cells) (Finnerup and Jensen, 2004; Yezierski, 2009). Although direct, injury-induced excitation of pain pathways should wane with time after SCI, the intense early activity is probably propagated widely in the CNS, and in different parts of pain pathways it might induce long-term synaptic alterations similar to those underlying long-term memory in the brain, such as late-phase long-term synaptic potentiation (LTP) (Asiedu et al., 2011; Laferriere et al., 2011; Marchand et al., 2011; Rahn et al., 2013). Indeed, memory-like synaptic alterations have been implicated in spinal neurons after SCI, which might contribute to the maintenance of chronic central sensitization and consequent pain (Crown et al., 2006; Tan and Waxman, 2012; Tan et al., 2008) (Table 1). Intense activation of neural pathways during SCI might also trigger long-term changes in ion channel function and increases in intrinsic excitability of neurons in pain pathways, for example by increasing the expression of voltage-gated Na^+ channel subunits such as Nav1.3 (Hains et al., 2003, 2005) or expression of the α2-δ1 voltage-gated Ca^{2+} channel subunit (Boroujerdi et al., 2011). Both of these molecules have been strongly implicated as necessary for the maintenance of SCI pain; SCI-induced reflex hypersensitivity was reversed by targeted molecular interventions (intrathecal injection of specific antisense oligodeoxynucleotides) that reduced the expression of each protein (Table 1). Importantly, enhanced neural activity resulting from neuronal hyperexcitability and synaptic potentiation would be expected to promote central neuroinflammation (Xanthos and Sandkuhler, 2014), so SCI-induced neural activity is likely to both drive and be driven by the neuroinflammatory mechanisms discussed in the following sections (see Fig. 1).

Other direct effects of SCI are anatomical, including the transection or demyelination of descending and ascending axons in the cord. These effects are likely to have different consequences at, below, and above the spinal injury level. Below-level pain after partial interruption of axonal tracts has been attributed to a loss of descending inhibition onto below-level nociceptive pathways so that activity in surviving ascending pain pathways is persistently increased (Bruce et al., 2002; Hains et al., 2002; You et al., 2008). Below-level behavioral hypersensitivity has also been associated with reduced activity in local GABAergic inhibitory interneurons in the spinal dorsal horn (Drew et al., 2004; Gwak et al., 2006), and with an apparent apoptotic loss of GABAergic neurons in lumbar segments relatively distant from a contusion injury at T11 (Meisner et al., 2010). Interruption of ascending pain pathways might also promote neuropathic pain; for example, deafferentation by SCI may cause increased sensitivity of pain-related neurons in the thalamus to other inputs (Wang and Thompson, 2008) and activity-dependent reorganization of cortical pain networks (Nardone et al., 2013).

SCI-induced changes in the balance of excitatory and inhibitory influences at any level of the pain pathway may result in neuropathic pain (Finnerup and Jensen, 2004; Whitt et al., 2013), but whether and how such changes may selectively impact pain felt at different levels are not clear. It has been suggested that a progressive buildup of hyperexcitability in neurons below a lesion eventually activates residual spinothalamic tract neurons sufficiently to drive below-level pain (Wasner et al., 2008). This possibility is supported by evidence that functional pathways can remain even after "complete" SCI (Detloff et al., 2008; Finnerup et al., 2004; Wasner et al., 2008). Evidence also supports the possibility that neuroinflammation below an injury promotes below-level neuronal hyperexcitability (Detloff et al., 2008; Hains and Waxman, 2006 Zhao et al., 2007b;). Anatomical changes in the spinal cord that might promote neuropathic pain include the sprouting of primary nociceptors, which have been observed primarily but not exclusively below the injury level (Krenz and Weaver, 1998; Ondarza et al., 2003). Interestingly, SCI triggers an enhanced, intrinsic growth state in primary nociceptors (Bareiss et al., 2013; Bedi et al., 2012). This enhanced growth state has been observed in neurons dissociated from dorsal root ganglia (DRG) below and at a thoracic injury level, but not above the level (Bedi et al., 2012), paralleling the distribution of other alterations in these sensory neurons (Bedi et al., 2010) and the bodily regions manifesting neuropathic pain in humans after SCI (e.g., Finnerup et al., 2014). Another anatomical alteration caused by SCI is the disruption of the blood-spinal cord barrier (BSCB) in the

Table 1
Mechanism-directed interventions reported to reduce long-lasting, pain-related behavior in animal SCI models.

Intervention/(mechanistic target)	Behavior reduced	Selected references
Nav1.3 knockdown i.t. (dorsal horn neuron hyperexcitability)	Hindlimb hypersens (mech and heat)	Hains et al. (2003)
Rac1 inhibitor i.t. spinal synaptic plasticity)	Hindlimb hypersens (mech and heat)	Tan et al. (2008)
av α2-δ1 knockdown i.t. (spinal synaptic transmission)	Hindlimb hypersens (mech)	Boroujerdi et al. (2011)
IL-10 delivery i.p., or HSV s.c. (inflammation, neuroinflammation)	Excessive grooming Hindlimb hypersens (mech and heat) Evoked pain (operant)	Plunkett et al. (2001), Lau et al. (2012)
COX-2 (i.p.), PGE2 (i.t.) inhibitors (inflammation, nueroinflammation)	Hindlimb hypersens (mech and heat)	Hains et al. (2001), Zhao et al. (2007a)
COX–LOX inhibitor oral (inflammation)	Hindlimb hypersens (mech)	Dulin et al. (2013)
CCL21 antibody local injection (thalamic microglial activation)	Hindlimb hypersens (mech and heat)	Zhao et al. (2007b)
TNFα blocker i.t. (neuroinflammation)	Hindlimb hypersens (mech)	Marchand et al. (2009)
Connexin 43 knockout (astrocyte coupling)	Hindlimb hypersens (mech)	Chen et al. (2012)
IL-6 receptor antibody i.p. (Neuroinflammation)	Hindlimb hypersens (mech)	Guptarak et al., 2013
TrkB.T1 knockout (glial BDNF signaling)	Hindlimb hypersens (mech)	Wu et al. (2013b)
TRPV1 knockdown i.t., inhibitor i.p. (nociceptor sensitization)	Hindlimb hypersens (mech and heat)	Wu et al. (2013c)

Notes. Only interventions in which the molecular targets and functional consequences are relatively well defined are included. See text for discussion of studies using other interesting agents, including commonly used drugs, such as minocycline, that have less defined targets and actions. Neuroinflammation is indicated as a mechanistic target if the delivery method primarily targets the nervous system. Abbreviations: i.p., intraperitoneal delivery, i.t., intrathecal; s.c., subcutaneous; HSV, herpes simplex vector; hypersens, reflex hypersensitivity; mech, mechanical.

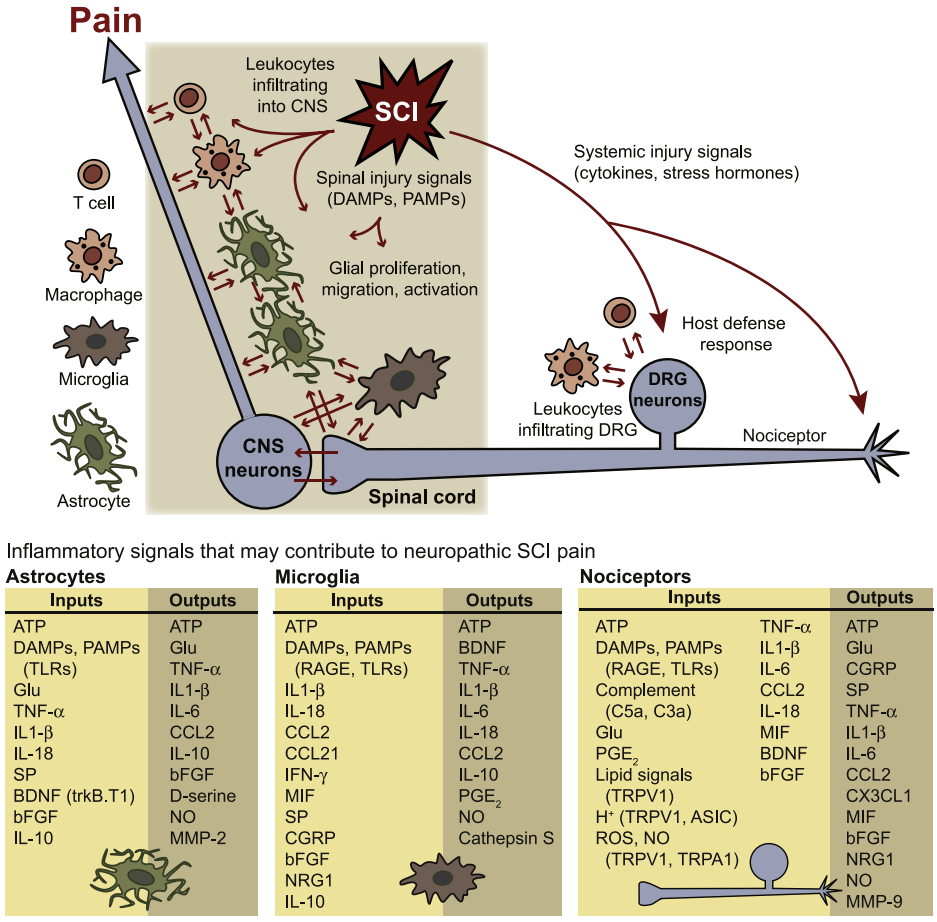

Fig. 1. Extracellular signaling involved in SCI-induced neuroinflammation and neuropathic pain, including a nociceptor-mediated link between peripheral host defense and central inflammation. The highly schematic diagram depicts some of the complex interactions among glial cells, infiltrating leukocytes (only macrophages and T cells are indicated, but other types are also involved), and neurons that have been implicated in the development and maintenance of neuropathic SCI pain. Spinal cord and dorsal root ganglia (DRG) are shown, but some of the interactions important for pain occur in supraspinal components of the pain pathway as well (see text). Listed below the schematic diagram are some of the inflammatory signals reported to stimulate (inputs) or be released by (outputs) each of three cell types prominently involved in neuroinflammation and neuropathic pain (evidence from both central and peripheral injury models): astrocytes, microglia, and nociceptors. Note that each list is incomplete and that some of the listed signals are not constitutively present, instead being conditionally induced in a given cell type (often by inflammation or injury). Some of the inflammatory signaling listed under nociceptors also occurs in central neurons. Signal abbreviations are defined in the text.

region of injury (Noble and Wrathall, 1987; Popovich et al., 1996). The disruption of the BSCB has been associated with pain after peripheral injury or inflammation (Beggs et al., 2010; Brooks et al., 2005), perispinal inflammation (Tenorio et al., 2013), and SCI (Lin et al., 2012). Among other effects, the disruption of the BSCB will permit blood-borne myeloid and lymphoid immune cells to enter the spinal cord parenchyma and exert direct inflammatory actions on central neurons and glia (Skaper et al., 2012; Zhang et al., 2011) (Fig. 1). An interesting possibility is that the disruption of the BSCB could also allow blood-borne factors generated below a spinal lesion to enter the CNS at and above a spinal injury, permitting below-level neural and glial activity to influence pain processing even when ascending pain pathways have been effectively interrupted.

Neuroinflammatory mechanisms of neuropathic SCI pain

Why spinal neuroinflammation should produce neuropathic SCI pain

Neuroinflammation is commonly and simply defined as an inflammation of part of the nervous system. Inflammation is classically defined as a coordinated response of the innate immune system that combats infection. The relatively primitive innate immune system is the first line of defense against pathogens and toxins; it is always present and it depends upon diverse cell types that include barrier cells, phagocytes, and various parenchymal cells in different tissues, including the nervous system. Unlike the more recently evolved adaptive immune system, the innate immune system does not employ antigen-specific humoral and cell-mediated immunity mechanisms. Two innate immune functions have been emphasized traditionally: 1) the recruitment of cells and proteins to destroy pathogens and toxins, and 2) increases in the flow of lymph containing pathogens, toxins, and antigen-presenting cells to lymphoid tissues to help initiate the more delayed adaptive immune response (Murphy, 2012). However, it has become increasingly evident that multiple inflammatory states exist, ranging from full-scale inflammation to mild, basal inflammatory responses, and these can be either transient or chronic. The wide range of inflammatory states probably covers numerous functions, including not only host defense but tissue repair and homeostatic adjustments to stress (Medzhitov, 2008). It is also clear that inflammation functions to repair injured tissue, including the nervous system (Benowitz and Popovich, 2011; Xanthos and Sandkuhler, 2014). Cells of the innate immune system are thus important for neuroinflammation and associated tissue repair. Many of these cells are in the myeloid lineage, including macrophages, granulocytes, dendritic cells, and mast cells. Particular emphasis has been placed by the investigators of pain on the myeloid cells residing in the CNS parenchyma that are closely related to macrophages–microglia (Calvo and Bennett, 2012; Ginhoux et al., 2010;

Graeber and Christie, 2012; Schomberg and Olson, 2012; Taves et al., 2013). However, inflammation involves responses to pathogens and tissue damage that are mediated by the interactions of myeloid immune cells with various other cell types, including endothelial cells and neurons. Non-myeloid cells in the CNS contributing to neuroinflammation that have received the most attention in pain studies are astrocytes. Indeed, interlinked, overlapping functions and assumed dysfunctions ("gliopathy") of astroglia and microglia appear to be especially important for neuropathic pain (Hulsebosch, 2008; Ji et al., 2013; McMahon and Malcangio, 2009; Watkins et al., 2001).

Five general observations suggest that SCI-induced neuroinflammation should produce pain. First, spinal inflammatory reactions induced by *peripheral* injury and/or inflammation promote pain-related behavior. In particular, several lines of evidence indicate that spinal neuroinflammation (activation, migration, and proliferation of microglia and astrocytes) induced by peripheral injury and inflammation can drive pain (reviewed by DeLeo and Yezierski, 2001; Watkins et al., 2001; Ji et al., 2013). Peripheral nerve injury can support central neuroinflammation by triggering infiltration of leukocytes into the cord (Skaper et al., 2012; Sweitzer et al., 2002) probably by enabling the disruption of the BSCB (Brooks et al., 2005; Gordh et al., 2006; Huber et al., 2001). In addition, peripheral injury or inflammation induces central neural activity that promotes neuroinflammation (Xanthos and Sandkuhler, 2014). Second, SCI causes the generation and widespread release of proinflammatory cytokines (e.g., Alexander and Popovich, 2009). Transient increases in TNFα and IL-1β as well as a persistent increase in IL-6 expression have been associated with behavioral indicators of SCI pain (Detloff et al., 2008). Third, proinflammatory cytokines can regulate synaptic transmission and plasticity in the spinal cord and brain. For example, TNFα and IL-1β can either enhance or suppress synaptic transmission and LTP by direct and indirect actions on glutamatergic synapses in the brain (including regulation of AMPA and NMDA receptor trafficking), and these complex effects on synaptic function may depend upon the degree or state of glial and neural activity (Beattie et al., 2002; Chu et al., 2012; del Rey et al., 2013; Perea and Araque, 2007; Stellwagen and Malenka, 2006; Yirmiya and Goshen, 2011). In the spinal cord, TNFα and IL-1β are reported to induce LTP at C-fiber synapses along with behavioral hypersensitivity both by direct actions on these synapses (Kawasaki et al., 2008) and by indirect actions that probably involve recurrent release of additional glial mediators (Gruber-Schoffnegger et al., 2013). Fourth, in uninjured animals, the intrathecal injection of proinflammatory cytokines, including IL-1β and IL-6, is sufficient to produce reflex hypersensitivity (DeLeo et al., 1996; Reeve et al., 2000). Fifth, increasing evidence indicates that neuroinflammation after SCI, like neuropathic SCI pain, persists indefinitely (Beck et al., 2010; Byrnes et al., 2011; Dulin et al., 2013a; Fleming et al., 2006; Nesic et al., 2005).

Given the general arguments just listed for why SCI-induced neuroinflammation should produce pain, a puzzle is why a substantial fraction of SCI patients with injuries indistinguishable from other SCI patients fails to report neuropathic pain (Dijkers et al., 2009). These dramatic differences within the SCI patient population in the severity of neuropathic pain emphasize the complexity of the systems involved. One intriguing factor could be the differential operation of endogenous pain suppression mechanisms, which may be recruited more effectively in some SCI patients than others.

Specific neuroinflammatory mechanisms that have been linked to neuropathic SCI pain are reviewed below, separated into mechanisms that have been associated thus far primarily with microglia and astrocytes. Bear in mind that this is a convenient simplification: some of these mechanisms occur in both types of glia, and many probably represent reactions that are promoted in one cell type by signals from the other glial cell type (and from non-glial cells). Fig. 1 depicts some of the complex extracellular signaling among microglia, astrocytes, and neurons that may be important for neuropathic SCI pain.

SCI pain mechanisms associated with microglia

The earliest direct evidence in animal models that inflammatory responses contribute to SCI pain came from the ability of interventions that reduce signaling by cells in the innate immune system, such as microglia and macrophages, to reduce reflex hypersensitivity after SCI. IL-10 is a potent anti-inflammatory cytokine that reduces the activation of many immune cells, including macrophages, microglia, and astrocytes, and is also produced by glia (Graeber and Christie, 2012; Thompson et al., 2013). Systemic injection of IL-10 shortly after excitotoxic spinal injury delayed the onset of and decreased the amount of excessive grooming caused by this type of injury, associated with a reduction both in neural damage and in signs of neuroinflammation (including reduced spinal expression of IL-1β, COX-2, and iNOS) (Plunkett et al., 2001) (Table 1). Moreover, knockout of IL-10 accelerated the onset of pain-related behavior and expansion of the lesion (Abraham et al., 2004). Recently, IL-10 delivered by a herpes simplex virus (HSV) vector after spinal contusion injury decreased spinal TNFα expression and astrocyte activation assessed by glial fibrillary acidic protein (GFAP) immunochemistry. Hindlimb mechanical and heat hypersensitivity were reduced by the IL-10 delivery and, importantly, so was an operant measure of evoked pain (Lau et al., 2012) (Table 1). These pain suppressive effects are consistent with the direct actions of IL-10 on activated microglia, although many other cell types may have contributed to IL-10's effects in these studies.

Widespread activation of microglia (spinal and supraspinal) occurs after SCI, as indicated by a shift in morphology from a ramified to ameboid shape and upregulation of the microglial markers CD11b/c/CCR3 (OX-42), major histocompability complex II (MHC II), or ionized calcium-binding adaptor molecule-1 (Iba-1) (e.g., Detloff et al., 2008; Dijkstra et al., 2000; Koshinaga and Whittemore, 1995; Nesic et al., 2005; Popovich et al., 1997; Schwab et al., 2005; Watanabe et al., 1999). The concept of glial activation or reactivity probably involves multiple states and functions in microglia and other myeloid cells (Hawthorne and Popovich, 2011), but these complications have largely been ignored thus far in studies of microglial contributions to SCI pain. Proliferation and migration of microglia also occur (Byrnes and Faden, 2007; Zai and Wrathall, 2005). Because of the prominent roles of macrophages in peripheral inflammation, and of the many proinflammatory signals secreted by both macrophages and microglia on other examples of pain-related behavior (Ellis and Bennett, 2013; Kettenmann et al., 2011; Ramesh et al., 2013), microglia were prime candidates to drive SCI pain. This possibility was strongly supported by the demonstration that spinal injection of microglia that had been activated by ATP was sufficient to produce mechanical hypersensitivity in uninjured animals (Coull et al., 2005).

Contributions of microglia to neuropathic pain after SCI have largely been studied using the tetracycline antibiotic, minocycline, to inhibit microglial activation. Although minocycline remains an effective and popular inhibitor of microglia, it has many other reported effects (Garrido-Mesa et al., 2013), which limit the conclusions that can be drawn about how it may reduce SCI pain. These include other actions that could also inhibit pain-related mechanisms, such as inhibition of matrix metalloproteinases (Matsumoto et al., 2009), scavenging of free radicals (Ulgen et al., 2011), inhibition of poly(ADP-ribose) polymerase-1 (PARP-1) (Alano et al., 2006), and inhibition of voltage-gated Na^+ channels (Kim et al., 2011). Because the critical molecular targets of minocycline have not yet been identified in SCI pain studies, minocycline is not included in Table 1. The first use of minocycline and the first explicit test of microglial involvement in SCI pain were reported less than a decade ago by Hains and Waxman (Hains and Waxman, 2006). One month after thoracic contusive SCI, intrathecal infusion of minocycline reduced the signs of microglial activation (decreasing P-p38 MAPK in OX-42-positive cells and reducing the number of OX-42-positive cells with an activated morphology), reduced spontaneous and evoked activity in lumbar dorsal horn neurons,

completely reversed heat hypersensitivity of hindlimb reflexes, and largely reversed mechanical hypersensitivity of the same reflexes. These investigators then provided evidence that microglia contribute to SCI-induced reflex hypersensitivity by an ERK1/2-dependent release of PGE2 and probable binding to neuronal EP2 receptors in the lumbar dorsal horn (Zhao et al., 2007b). In vivo application of an inhibitor of p38-MAPK, SB203580, reduced supraspinally mediated behaviors (vocalization, biting, escape) to at-level mechanical stimulation after contusive SCI (Crown et al., 2008). One difference in this study was that evidence for increased P-p38-MAPK was found not only in microglia, but also in astrocytes and neurons. A recent study found enhancement by SCI of P-p38 MAPK and PGE2 production that could be ameliorated by acupuncture (Choi et al., 2012). Zhao et al. (2007a) showed a strong suppression of SCI-induced hindlimb hypersensitivity by the intrathecal injection of an EP2 receptor antagonist (Table 1), while an earlier study had shown that systemic application of a COX-2 inhibitor reduced mechanical and heat hypersensitivity in hindlimbs and forelimbs associated with decreased PGE2 levels in the cord after contusive SCI (Hains et al., 2001) (Table 1). However, the potential clinical significance of the COX-2/PGE2 findings can be questioned because nonsteroidal anti-inflammatory drugs that primarily inhibit COX activity are not very effective in relieving neuropathic pain after SCI in humans (Cardenas and Jensen, 2006). On the other hand, neuroinflammation is associated with elevated arachidonic acid (AA) metabolism, and AA is metabolized not only to prostaglandins by COX but also to leukotrienes by 5-lipoxygenase (LOX). Interestingly, leukotrienes, like prostaglandins, contribute to the behavioral hypersensitivity in other pain models (Noguchi and Okubo, 2011). A recent study (Dulin et al., 2013a) showed that both the proinflammatory leukotriene B4 and PGE2 were elevated at a spinal contusion site 9 months after injury, and that treatment of rats for 1 month starting 8 months after SCI with a dual COX/5-LOX inhibitor, licofelone, reversed mechanical hypersensitivity of the hindpaws (Table 1). If licofelone is also found to reverse operant measures of pain in rodents, it would be one of the more promising potential treatments for neuropathic SCI pain to be suggested by preclinical studies.

Evidence for supraspinal microglial contributions to neuropathic SCI pain has also been found. Spinal contusion injury increased OX-42 expression in cells in the ventral posterolateral (VPL) nucleus in the thalamus and increased neuronal levels of a chemokine, CCL21, in the thalamus and spinal cord (Zhao et al., 2007a) (see also Wu et al., 2013a). Electrical stimulation of the spinothalamic tract also increased CCL21 in the thalamus. Intra-VPL injection of CCL21 increased microglial activation and increased hindlimb mechanical and heat sensitivity, whereas both a neutralizing antibody to CCL21 and minocycline reversed the SCI-induced behavioral hypersensitivity as well as the microglial and neuronal effects produced in the VPL (Zhao et al., 2007a) (Table 1). These results suggest that pain-promoting activation of supraspinal neurons is driven by activity-dependent release of at least one chemokine, CCL21, from spinothalamic tract neurons after SCI.

Some SCI studies differ from those just described in suggesting that microglial activity is more important for the early induction than the later maintenance of neuropathic pain, as was previously indicated by studies using peripheral neuropathic pain models (Ledeboer et al., 2005; Owolabi and Saab, 2006; Raghavendra et al., 2003; Zhuang et al., 2005). Electrolytic lesion of the spinothalamic tract that caused persistent mechanical and heat hypersensitivity was associated with early, transient upregulation of Iba1 followed by long-lasting upregulation of GFAP (Naseri et al., 2013). Early administration of minocycline (beginning minutes after injury and repeated for 2–5 days afterwards) attenuated the development of mechanical and heat sensitivity as shown by reflex tests 1 and 2, weeks after spinal hemisection, (Marchand et al., 2009) or tests 4, weeks after spinal contusion (Tan et al., 2009). Thus, early administration of minocycline, unlike later treatments (Hains and Waxman, 2006; Zhao et al., 2007b), produced reductions in behavioral hypersensitivity that long outlasted the drug application. TNFα, a proinflammatory cytokine that is synthesized and released by various cell types, including neurons and astrocytes, but in the CNS primarily by microglia (Kraft et al., 2009), plays a particularly important role in the initiation of inflammatory cascades (Montgomery and Bowers, 2012). Interestingly, etanercept, a fusion protein blocker of TNFα, dramatically reduced behavioral hypersensitivity 1 to 4 weeks after spinal hemisection if given for 2 days beginning immediately after injury, but had no effect when delivered 2 to 3 weeks after injury (Marchand et al., 2009) (Table 1). These observations are consistent with early signaling by reactive microglia promoting a longer-lasting activation of astrocytes (e.g., Giulian et al., 1994; Lee et al., 1993; Raghavendra et al., 2003) that helps to maintain chronic SCI pain.

SCI pain mechanisms associated with astrocytes

Although derived from neuroepithelial rather than the myeloid or lymphoid progenitors of immune cells, astrocytes are essential participants in neuroinflammation, which depends upon interactions among astrocytes, microglia (and sometimes other host defense cells), and neurons (Alexander and Popovich, 2009). Indeed, many investigators consider astrocytes to be part of the innate immune system (Ransohoff and Brown, 2012). Numerous studies have indicated that astrocytes make major contributions to pain-related behavior following *peripheral* nerve injury and inflammation, and this extensive body of work is covered comprehensively by several recent reviews (Ellis and Bennett, 2013; Ji et al., 2013; Mika et al., 2013). Importantly, spinal injection of astrocytes that had been activated by TNFα was shown to be sufficient to produce mechanical hypersensitivity in uninjured animals (Gao et al., 2010). Far fewer studies have been made of astroglial contributions to neuropathic pain caused by SCI, but the findings thus far show interesting similarities to what has been described in peripheral neuropathic pain models. Like microglia, astrocytes proliferate after SCI, especially in the region of the lesion, forming a glial scar (Byrnes and Faden, 2007; Karimi-Abdolrezaee and Billakanti, 2012; Zai and Wrathall, 2005). This proliferation and the activation of astroglia (reactive gliosis) by SCI (e.g., Baldwin et al., 1998; Carlton et al., 2009; Gwak and Hulsebosch, 2009; O'Brien et al., 1994; Popovich et al., 1997) may be even more pronounced than the various forms of activation reported for microglia after SCI, as has also been noted in models of peripheral injury and inflammation (Ji et al., 2013). Reactive gliosis is typically assessed by the upregulation of GFAP, which is a rapid and relatively specific indicator of astrocyte activation. Using both immunostaining and Western blot to measure GFAP expression, an early study showed that thoracic spinal contusion caused astrocyte activation not only at the lesion site but also throughout the spinal cord, and striking increases in GFAP remained for as long as the investigators assayed the cord (up to 9 months after SCI) (Nesic et al., 2005). Importantly, this same study showed that the changes in GFAP expression were correlated with mechanical hypersensitivity of hindpaws and forepaws, and showed that other proteins preferentially expressed in astrocytes or likely to be involved in pain-related functions of reactive astrocytes were also upregulated a month or later after SCI. These included the astrocytic Ca^{2+}-binding protein S-100 and the water channel protein aquaporin 4 (implicated in inflammatory edema in the CNS, which probably involves the extensive contacts made by astrocytes onto spinal blood vessels, Fukuda and Badaut, 2012). Whereas some studies (Carlton et al., 2009; Gwak et al., 2012; Nesic et al., 2005) have reported persistent changes in GFAP expression in segments distant from a thoracic injury site associated with changes in behavioral hypersensitivity, other studies have reported a lack of immunohistochemical or morphological evidence for astrocyte activation in some of the same spinal segments after similar traumatic SCI (Andrews et al., 2012; Detloff et al., 2008). Thus, while changes in astrocyte function might be involved in SCI-induced pain, prominent changes in astrocyte morphology and GFAP expression in lumbar segments do not appear to be necessary for at least one behavioral indicator of allodynia after SCI — hindlimb hypersensitivity (Detloff et al., 2008).

Recent evidence suggests that activated astrocytes may promote allodynia by increasing production of IL-6 (Guptarak et al., 2013).

Efforts to define contributions of astrocytic mechanisms to SCI pain have been limited by a lack of specific inhibitors of astrocyte activity. Preclinical studies of SCI pain have described the suppression of reflex hypersensitivity by drugs that target astrocytes but also microglia and other cell types, notably propentofylline (Gwak and Hulsebosch, 2009; Gwak et al., 2008, 2009) and also ibudilast (Hama et al., 2012). In peripheral nerve injury models, pain has been associated with relatively high activity of c-Jun N-terminal kinase (JNK) in astrocytes compared to neurons or microglia (Zhuang et al., 2006). Recently it was reported that contusive SCI at T10 caused the activation of JNK in lumbar segments lasting at least a month, and that mechanical and heat hypersensitivity of hindlimb reflexes was reduced by the intrathecal injection of the JNK inhibitor, SP600125 (Lee et al., 2013). Interestingly, the same study found similar suppressive effects produced by acupuncture. Perhaps the most compelling evidence for essential roles of astrocytes in neuropathic SCI pain has come from the investigations of a notable feature of astrocytes that is absent or sparse in microglia: coupling by gap junctions containing connexin 43 (Cx43). Transgenic mice with deletion of Cx43 showed reduced GFAP expression 1–2 months after contusive SCI and little or no mechanical or heat hypersensitivity of paw withdrawal (Chen et al., 2012) (Table 1). Interestingly, this study showed that the suppression of the persistent hypersensitivity was much more dramatic after Cx43 knockout than after early minocycline treatment (similar to that used in rats by Marchand et al., 2009 and Tan et al., 2009), suggesting a potentially larger role for reactive astrocytes than microglia in the development of neuropathic SCI pain, at least in mice. Consistent with a major role for intracellular coupling among astrocytes in the initial development of SCI pain, early but not late treatment of rats with the gap junction decoupler, carbenoxolone, reduced later mechanical and heat hypersensitivity, as well as GFAP staining after spinal hemisection (Roh et al., 2010).

Reactive gliosis after SCI persists for as long as it has been examined in animal models (Gwak et al., 2012; Wu et al., 2012). Inhibition of proliferation in general (and possibly of other cellular effects in non-proliferating neurons) by a cyclin-dependent cyclase (CDK) inhibitor, CR8, was found to produce parallel reductions in lesion volume, in SCI-induced upregulation of cell cycle-related proteins in astrocytes and microglia, in the elevated expression of GFAP and Iba-1, and in various signs of central inflammation months after SCI (Wu et al., 2012). Interestingly, these effects and concomitant neuropathic SCI pain appear to depend upon a neurotrophin, BDNF, that is associated with many forms of neural plasticity, including pain-related plasticity in peripheral injury models (Merighi et al., 2008), and which prominently involves ATP-induced release of BDNF from microglia (Coull et al., 2005; Trang et al., 2011). Neuronal effects of microglial BDNF release mediated by trkB receptors have been emphasized in peripheral neuropathic pain models. However, BDNF effects are also mediated through an alternatively spliced truncated form of the BDNF receptor, trkB.T1, which is expressed in astrocytes, oligodendrocytes, and Schwann cells as well as in neurons (Fenner, 2012) and has been shown to be important for neuropathic SCI pain. An extensive study (Wu et al., 2013b) found an upregulation of trkB.T1 for at least 2 months at a thoracic contusion site (see also Liebl et al., 2001) and at least 3 days in the distant lumbar enlargement in wild-type mice. Mice with genetic deletion of trkB.T1 showed a significant reduction of mechanical hypersensitivity in hindlimb tests (Table 1), which was associated with enhanced recovery of motor function, a smaller lesion volume, less GFAP and Iba-1 expression, a persistent downregulation of genes and proteins in cell cycle pathways, and less elevation of cell cycle-related protein expression in reactive astrocytes assayed with an in vitro model system. Deletion of trkB.T1 occluded the suppressive effects of the CDK inhibitor on mechanical hypersensitivity and motor recovery after SCI (Wu et al., 2013b). These intriguing findings point to important roles for BDNF, trkB.T1, and cell cycle-related proteins in neuropathic SCI pain, but much remains to be learned about where and how these molecules contribute to neuroinflammation, glial proliferation, and pain after SCI.

In sum, observations from SCI pain models, supported by a larger body of similar evidence from peripheral neuropathic pain models (e.g., Colburn et al., 1999; Sweitzer et al., 1999; Zhuang et al., 2005) indicate that interactions among astrocytes, microglia, and neurons are critical for the development and maintenance of neuropathic SCI pain (Gwak et al., 2012; Ji et al., 2013). Important interactions may also involve satellite glial cells in sensory ganglia, which are closely related to astrocytes and are known to contribute to behavioral hypersensitivity in peripheral models of neuropathic pain (Huang et al., 2013; Ji et al., 2013; Ohara et al., 2009; Xie et al., 2009), but contributions of satellite glial cells to painful consequences of SCI have yet to be reported.

Is neuropathic SCI pain driven by a unified host defense system?

An implicit assumption guiding most work on neuropathic SCI pain is that the pain arises in a disorganized fashion from any of numerous, often independent effects of the injury on different components of pain pathways (see section above on classes of mechanisms). An interesting possibility is that SCI also inadvertently activates integrated response systems that employ pain and neuroinflammation as part of host defense against pathogens, parasites, and other threats. It has long been appreciated that many interactions occur between the innate immune system and the peripheral nervous system, with peripheral neurogenic inflammation being especially prominent (Richardson and Vasko, 2002; Xanthos and Sandkuhler, 2014). Compelling arguments have been made for viewing the immune and somatosensory nervous systems as composing a unified system that functions in host defense, broadly conceived as integrating perceptual and behavioral responses (pain behavior) with classical responses of the innate and adaptive immune systems (Chiu et al., 2012). By this view, primary nociceptors (which strongly activate pain pathways) are the first responders for host defense, initiating not only rapid perceptual and behavioral responses but also early inflammatory responses (neurogenic inflammation) to tissue injury and infection. This broad sentinel role accounts for the expression in C-fiber nociceptors of numerous pattern recognition receptors (PRRs) for damage-associated molecular patterns (DAMPs or alarmins: intracellular molecules such as high-mobility-group box 1 [HMGB1] and heat shock proteins released by ruptured or necrotic cells) and pathogen-associated molecular patterns (PAMPs, e.g., components of bacterial and yeast cell wall components and viral RNA), as well as receptors for intense mechanical stimulation and for signs of ongoing inflammation (Fig. 1). In C-fiber nociceptors under normal, inflamed, and/or injured conditions these often include TRP channels (especially TRPV1 and TRPA1), cytokine and prostaglandin receptors (e.g., IL-1β R, TNFα-R, IL-6-R, EP4), chemokine receptors (e.g., CCR2 for MCP-1/CCL2), PRRs (e.g., toll-like receptors [TLRs] 3, 4, 7, and 9, Nod-like receptors [NLRs] and receptor for advanced glycation endproducts [RAGE]), receptors for other intracellular constituents released during injury (P2X3 and P2Y ATP receptors, AMPA and NMDA glutamate receptors), and receptors for growth factors (e.g., trkA for nerve growth factor [NGF] and trkB for BDNF) (Chiu et al., 2012; Miller et al., 2009; Shibasaki et al., 2010). In addition, primary nociceptors can be sensitized and activated by other effector molecules in the innate immune system important in inflammation, including the complement fragments C5a and C3a, suggesting the presence of complement receptors on these neurons (Jang et al., 2010). Most, if not all, of these diverse receptors are expressed on peripheral and central terminals, where they modulate the gain of nociceptor inputs and outputs, respectively, and also on nociceptor somata in the DRG, where they may detect blood-borne danger signals unimpeded by a significant vascular permeability barrier (Abram et al., 2006; Jimenez-Andrade et al., 2008). Primary nociceptors communicate directly with cells in the innate immune system (including peripheral macrophages, neutrophils, and T cells, and central microglia

and astrocytes) by releasing glutamate, ATP, neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (SP), chemokines such as CCL2, CCL21, and CX3CL1 (fractalkine), cytokines such as IL1β, IL-6, and TNFα, and growth factors such as BDNF, neuregulin 1 (NRG1), and basic fibroblast growth factor (bFGF) (Calvo and Bennett, 2012; Chiu et al., 2012; Ji et al., 2013; McMahon and Malcangio, 2009; Miller et al., 2009; Pezet and McMahon, 2006) (Fig. 1). An integral role of primary nociceptors in innate immunity recently received unexpected affirmation by the discovery that bacteria can directly activate these neurons without mediation by known immune cells (Chiu et al., 2013).

This broader biological view raises the interesting possibility that SCI generates a complex set of signals that are detected by primary nociceptors, which if then sufficiently activated, drive pain as part of a unified host defense response. A complementary view is that persistent central and peripheral signals generated by SCI mimic the pattern that would be generated by a very severe peripheral injury, and this induces a long-lasting hyperfunctional state in numerous primary nociceptors that would normally serve to compensate for the loss of peripheral sensory function and to protect body regions made more vulnerable by locally disabling injury (Walters, 2012). In particular, nociceptors may be sensitive to central as well as peripheral inflammatory signals (integrating these with other signals of severe bodily injury, such as retrograde signals from intensely activated postsynaptic neurons, Walters, 2012), and nociceptor activity may in turn stimulate central as well as peripheral inflammatory responses (see below). An implication of these views is that positive feedback loops between enhanced electrical activity in primary nociceptors and combined activation of peripheral and central inflammatory cells may help to sustain neuroinflammation and chronic neuropathic pain (Miller et al., 2009; Walters, 2012; Xie et al., 2009).

Support for these views first came from evidence that contusive SCI enhances the growth of uninjured nociceptors distant from a spinal lesion site (Bedi et al., 2012; Hou et al., 2009; Krenz and Weaver, 1998; Ondarza et al., 2003; Ramer et al., 2012). SCI was then found to enhance peripheral function in C-fiber nociceptors, with sensitivity to mechanical and heat stimuli being increased in a forepaw skin-nerve preparation 5 weeks after T10 contusion (Carlton et al., 2009). Significantly, this study found that spontaneous electrical activity (SA) was generated at a low rate in the peripheral terminals of nociceptors after SCI. Nociceptor SA induced by SCI was also found to be generated in the soma in the DRG in vivo and in vitro (Bedi et al., 2010). Intrinsic SA and hyperexcitability were present in ~50% of small neurons dissociated from DRGs below and at (but not above) the T10 injury level, and the high incidence remained unchanged for at least 5 months after SCI. Importantly, the intrinsic SA was correlated with mechanical and heat hypersensitivity of hindlimb and forelimb withdrawal responses, as well as with increased incidence of a supraspinally mediated response, vocalization, evoked at but not below the injury level (Bedi et al., 2010) — a pattern like that reported by many SCI patients (Finnerup et al., 2014).

Most of the dissociated DRG neurons showing SA after SCI were responsive to the specific activator of TRPV1, capsaicin (Bedi et al., 2010), and TRPV1 expression was increased in lumbar DRGs 4–6 weeks after thoracic contusion (Wu et al., 2013c) (see also DomBourian et al., 2006; Ramer et al., 2012; Zhou et al., 2002). Very low concentrations of capsaicin (10 nM) produced non-desensitizing, non-accommodating repetitive firing in dissociated nociceptors indistinguishable from SCI-induced SA, and this effect and other cellular responses to capsaicin were enhanced by prior SCI (Wu et al., 2013c). Most important, SCI-induced mechanical and heat hypersensitivity of hindlimb withdrawal responses was reversed by antisense knockdown of TRPV1 or by systemic injection of a specific TRPV1 antagonist, AMG9810 (Wu et al., 2013c) (Table 1) (see also Rajpal et al., 2007). While TRPV1 channels have been observed in other cells, they are expressed most abundantly in nociceptors (Caterina et al., 2000; Lauria et al., 2006), supporting the possibility that interruption of nociceptor SA contributed to these suppressive effects. A major role for nociceptor activity after SCI was also indicated by reversal of SCI-induced reflex hypersensitivity by knockdown of a voltage-gated Na^+ channel, Nav1.8 (Yang et al., 2012) that is primarily expressed in primary somatosensory neurons, including >90% of C-fiber nociceptors (Liu and Wood, 2011; Shields et al., 2012). TRPV1 has important functions in host defense, being activated and/or sensitized by many features of inflammation, including acidity, numerous lipids generated during cellular injury or ischemia, and a growing number of other injury-related molecules reported (amines, ATP, NO, reactive oxygen species [ROS], CCL2) (Jung et al., 2008; Miyamoto et al., 2009; Morales-Lazaro et al., 2013; Nishio et al., 2013). Thus, after SCI, TRPV1 receptors may detect multiple signs of neuroinflammation both peripherally and in the spinal cord — specifically, in the central processes of nociceptors and/or in TRPV1-expressing spinal neurons (Kim et al., 2012). Interestingly, evidence in mouse models of peripherally induced pain indicates that neuronal TRPV1 function contributes to the activation of both microglia and astroglia (Chen et al., 2009).

The somata of primary nociceptors may be an important locus for detecting inflammatory signals and integrating them with other signals of serious bodily injury (Walters, 2012). Pain-promoting sensitization of DRG neurons is known to occur after experimental inflammation around a ganglion (Wang et al., 2007; Xie et al., 2006), which causes an upregulation of TRPV1 in nociceptors and the generation of numerous cytokines in the DRG (Dong et al., 2012; Strong et al., 2012). After SCI, nociceptor somata are exposed to macrophages and T cells that infiltrate into DRGs close to and distant from a spinal lesion (McKay and McLachlan, 2004). The relatively ineffective vascular permeability barrier of DRGs (Abram et al., 2006; Jimenez-Andrade et al., 2008) and normal exposure of DRG neurons to cerebrospinal fluid (CSF) means that nociceptor somata will be highly exposed to the elevated levels of cytokines that have been observed in the blood (Davies et al., 2007; Stein et al., 2013) and CSF (Kwon et al., 2010) of SCI patients. One of these cytokines, macrophage migration inhibitory factor, MIF, is secreted by the leukocytes and the anterior pituitary gland, and is constitutively expressed in many other cells, including microglia and DRG neurons (Alexander et al., 2012; Bucala, 1996; Wang et al., 2010). MIF is particularly interesting because the circulating concentration of MIF is normally ~1000 fold higher than other pro-inflammatory cytokines (Aloisi et al., 2005; Bucala, 1996; Calandra and Roger, 2003), and this concentration was doubled in SCI patients compared to uninjured controls (Stein et al., 2013). Importantly, the concentration of MIF in SCI patients, ~1 ng/ml, was the same as shown to increase the excitability of a subset of putative nociceptors isolated from mouse DRGs (Alexander et al., 2012). This latter study also showed that MIF-null mice fail to develop pain after nerve injury or hindpaw inflammation, suggesting that MIF function is essential for both neuropathic and inflammatory types of pain, extending previous findings in rats (Wang et al., 2010, 2011). Furthermore, MIF application increased the expression of TNF-α, IL-1β, IL-6, CCL2, and iNOS in mouse and rat microglia, and MIF increased neurite outgrowth in isolated mouse DRG neurons (Alexander et al., 2012). All of these effects have also been observed after SCI (see above). The actions of MIF on microglia suggest that it could contribute to neuroinflammation and pain after SCI by central actions on microglia as well as by peripheral activation of nociceptors, raising the intriguing possibility that MIF has a key role in integrating painful neuroinflammatory responses of a unified host defense system to SCI. SCI also releases stress hormones (Fig. 1), such as glucocorticoids into the circulation, and these can result in immunosuppression, potentially opposing neuroinflammatory responses to SCI (Lucin et al., 2009). Interestingly, glucocorticoids induce MIF (Flaster et al., 2007), suggesting that the coordinated upregulation of MIF may function to preserve or enhance pain sensitivity during stressful conditions, such as SCI, when glucocorticoids suppress many other aspects of immune function

(Alexander and Popovich, 2009). Glucocorticoids and stress have also been shown to enhance neuropathic pain (Alexander et al., 2009).

Inflammatory responses to peripheral injury that occur both in DRGs and in the spinal cord depend upon electrical activity in primary afferent neurons (Hathway et al., 2009; Thacker et al., 2009; Van Steenwinckel et al., 2011; Wen et al., 2007; Xanthos and Sandkuhler, 2014; Xie et al., 2009). An important implication of this observation should be emphasized. If primary nociceptors function as part of the host defense system, then the central neuroinflammatory responses evoked by nociceptor activity may also represent a host defense function, at least under some conditions. Thus, from a broader biological perspective, central neuroinflammation may not always be maladaptive; limited nociceptor-evoked spinal neuroinflammation might, for example, be a mechanism that helps maintain adaptive pain targeted to a severely injured body part (Walters, 2012). Central neuroinflammation driven by nociceptor activity after SCI may be especially important in regions distant from a spinal injury site, where there would be much less damaged tissue generating DAMPs and other injury signals to drive local inflammation. Although it has been suggested that C-fiber nociceptors may be less important than other primary afferents for driving central neuroinflammation after peripheral nerve injury (Suter et al., 2009), this inference was based on sciatic nerve block methods that would not have reduced persistent SA generated in nociceptor somata in the DRG proximal to the block. Taken together, the studies reviewed in this section support the hypothesis that primary sensory neurons, including C-fiber nociceptors, are an integral part of a unified host defense system that can drive both peripheral (Chiu et al., 2012) and central inflammatory responses, and they support the possibility that this system may be activated after SCI to help drive neuropathic SCI pain (Fig. 1). Of course, the host defense system evolved to produce adaptive pain after peripheral injury and inflammation, so it should also be important for driving the second general class of pain endured by SCI patients — nociceptive pain triggered by overuse and by other secondary consequences of SCI for peripheral tissues.

Implications of neuroinflammatory mechanisms for treating neuropathic SCI pain

No front-line treatments currently used for neuropathic SCI pain specifically target neuroinflammatory mechanisms, although some probably do so indirectly. Standard treatments for SCI pain are based on those commonly (and with only limited success) used for peripheral neuropathic pain, notably the anticonvulsants gabapentin and pregabalin, and antidepressants such as amitriptyline, although many other drugs are used, including other serotonin–norepinephrine reuptake inhibitors, opioids, and intrathecal delivery of clonidine and ziconotide (Finnerup and Baastrup, 2012). Only pregabalin has been approved by the FDA for the treatment of neuropathic SCI pain, while other drugs used for this purpose were approved for other uses. Pregabalin (one target of which is the $\alpha2$-$\delta1$ voltage-gated Ca^{2+} channel subunit; see above and Table 1) has shown partial efficacy in two large-scale, randomized, placebo-controlled clinical trials (Cardenas et al., 2013; Siddall et al., 2006). Smaller randomized controlled trials have also indicated partial efficacy for gabapentin (Teasell et al., 2010), which shares mechanisms of action with pregabalin. A randomized controlled trial has shown significant but partial efficacy of amitriptyline in depressed but not non-depressed SCI patients (Rintala et al., 2007). Other commonly used drugs either have exhibited very little or no efficacy in clinical trials (e.g., lamotrigine), present major problems for long-term use (e.g., i.v. ketamine, i.v. lidocaine), or have not yet been tested rigorously in clinical trials for SCI pain (oral opioids, oral ketamine) (Teasell et al., 2010). Importantly, no drugs have demonstrated high efficacy against neuropathic SCI pain, and all have significant adverse side effects (Finnerup and Baastrup, 2012; Teasell et al., 2010).

Given the findings from animal models reviewed above, therapeutic approaches explicitly targeting neuroinflammatory mechanisms would be logical alternatives or complements to existing treatments for neuropathic SCI pain. Early evidence suggests that finding such treatments may be possible but challenging, as indicated by disappointing results in clinical trials for some of the agents that seemed quite promising in preclinical models of neuropathic pain. Treatment of patients with IL-10 (see also Table 1) illustrates general problems that can prevent the therapeutic use of an agent that effectively reduces neuroinflammation in both animal models and humans. On the basis of its powerful anti-inflammatory effects in a variety of animal models (including models of rheumatoid arthritis, diabetes, and inflammatory bowel disease), administration of IL-10 by direct injection, viral delivery, or adoptive transfer of IL-10-secreting cells appeared to offer exciting potential for treating many clinical conditions, including neuropathic pain (Milligan et al., 2012). However, IL-10 delivery by repeated injections in various clinical trials has failed to improve disease symptoms and has revealed serious adverse effects, including a marked reduction in red blood cell counts (Bijjiga and Martino, 2013). Furthermore, trials with gene therapy strategies that could produce more sustained elevations of IL-10 levels have not been attempted because of concern about potential dangers of prolonged immune suppression, including chronic infections and increased likelihood of certain cancers, as well as changes in cytokine balance that can increase allergic responses and asthma (Bijjiga and Martino, 2013). Moreover, a significant potential problem after SCI is that general suppression of inflammation may impair regeneration and repair in the spinal cord (Benowitz and Popovich, 2011). Nevertheless, the strategy of harnessing endogenous anti-inflammatory signals to combat neuropathic SCI pain is appealing. Anti-inflammatory, pro-resolution lipid signals such as resolvins, protectins/neuroprotectins, and lipoxins that have shown efficacy in preclinical models of peripheral neuropathic pain (Ji et al., 2011; Serhan et al., 2008) should offer promising candidates to investigate in the context of neuropathic SCI pain.

Other agents that reduce neuroinflammation and pain in rodent SCI models have either failed to alleviate neuropathic pain in clinical trials or have not yet been tested for effects on neuropathic pain. The nonspecific glial inhibitor, propentofylline, failed to decrease pain in postherpetic neuralgia patients (Lau et al., 2012). An unsettling note for preclinical studies of neuroinflammation is that this clinical failure may have reflected unexpected differences between human and rodent microglial properties, with human microglia being less responsive to a potent inflammogen, lipopolysaccharide (LPS), and to propentofylline than are rat microglia (Landry et al., 2012). Another nonspecific glial inhibitor, ibudilast, has demonstrated safety but only weak evidence of neuroprotection in a multiple sclerosis trial (Barkhof et al., 2010), and no results on efficacy against pain have been reported. In a small preliminary trial, the nonspecific microglial inhibitor, minocycline, failed to reduce pain caused by capsaicin application in patients with unilateral sciatica, but a trend was noted to improve ongoing pain prior to the capsaicin test (Sumracki et al., 2012). Larger clinical trials are underway to investigate minocycline's efficacy in treating pain associated with peripheral nerve damage (ClinicalTrials.gov: NCT01869907) and intercostal neuralgia (ClinicalTrials.gov: NCT01214482). Weak clinical results were also reported for a blocker of the chemokine, CCL2; an antagonist, AZD2423, of the CCR2 receptor showed no efficacy on primary pain variables after post-traumatic neuralgia, although somewhat encouraging trends were noted in subscores for paroxysmal pain and paresthesia/dysesthesia (Kalliomaki et al., 2013). Other plausible approaches to reduce neuroinflammation-associated pain have not yet been tested in clinical trials on neuropathic pain. Licofelone, which inhibits both COX and LOX enzymes in the arachidonic acid cascade, has shown effectiveness in clinical trials on arthritis, albeit with mixed results on pain (Raynauld et al., 2009; Wildi et al., 2010). Arthritic pain and central neuroinflammatory pain may differ in critical underlying mechanisms, so the possibility remains that neuropathic SCI pain will be sensitive to licofelone. A second potentially beneficial effect of licofelone was observed in a rodent SCI model: it reduced p-glycoprotein-mediated

drug resistance (Dulin et al., 2013b), suggesting that dual inhibitors of COX and LOX, such as licofelone, might simultaneously reduce neuroinflammation, ameliorate neuropathic pain, and improve bioavailability of other therapeutic drugs in SCI patients. Another potentially interesting target for therapeutic drug development is MIF, the inhibition of which may have potent effects on neuropathic pain (Alexander and Popovich, 2009), although a role in neuropathic SCI pain has yet to be demonstrated.

A novel but untested approach to treating neuropathic SCI pain may be to target ongoing activity in primary nociceptors. This strategy is suggested by indications that primary nociceptors are an integral part of a host defense system that can contribute to central as well as peripheral inflammation (and consequent pain), and by preclinical evidence that persistent activity in nociceptors drives behavioral hypersensitivity after SCI (Wu et al., 2013c; Yang et al., 2012) (Table 1). These preclinical studies found that interventions that reduced TRPV1 or Nav1.8 function sufficiently to eliminate spontaneous activity in nociceptors, but not to block reflex responses evoked by mechanical or heat stimuli, were effective in reversing behavioral hypersensitivity. This suggests that central sensitization and neuropathic SCI pain might be attenuated selectively by using more prolonged treatment with lower doses of TRPV1 antagonists (which, unlike Nav1.8 antagonists, have been tested successfully in humans) than tried thus far in clinical trials (Chizh et al., 2007; Krarup et al., 2011, 2013; Rowbotham et al., 2011; Schaffler et al., 2013). A new generation of potent and selective TRPV1 antagonists is becoming available (e.g., Reilly et al., 2012) that lack the hyperthermic side effects of previous antagonists (Gavva et al., 2008). If interventions that block ongoing activity of nociceptors and reduce reflex hypersensitivity are also found to block operant indications of chronic, ongoing pain in rodent models, this would provide a strong impetus for testing whether similar drugs reduce ongoing pain in SCI patients.

Conclusions

While multiple mechanisms contribute to neuropathic pain after SCI, numerous experimental observations indicate that persistent neuroinflammation is critical for the development and maintenance of this pain. Most of these observations are recent; only within the last decade has it become clear that neuroinflammation after SCI is chronic (perhaps permanent) and that interventions that target persistent neuroinflammation ameliorate behavioral hypersensitivity in animal models of neuropathic SCI pain. A limitation in nearly all of the animal studies is a reliance on spinally-mediated reflexive measures of pain, usually the enhancement of hindlimb withdrawal responses, which bear an uncertain relationship to the cortically-mediated states having emotional and cognitive components that are central to the human pain experience. Nevertheless, neuroinflammation and behavioral hypersensitivity after SCI have been linked to altered activity in several components of classical pain pathways that would be expected to promote pain, including primary afferent neurons, dorsal horn neurons, and thalamic neurons. Studies have shown correlations between SCI-induced reflex hypersensitivity and several measures of activation and proliferation by microglia (plus infiltrating macrophages) and astrocytes, along with parallel reversals of behavioral and glial alterations by interventions designed to reduce neuroinflammation. The interventions that have appeared successful in reducing SCI pain include treatments with agents that inhibit the activation of microglia and/or astroglia (IL-10, minocycline, etanercept, propentofylline, ibudilast, licofelone, SP600125, carbenoxolone), pharmacological (CR8) and/or genetic disruption of cell cycle-related proteins or a truncated receptor (trkB.T1) for BDNF, and reduction in the activity of channels (TRPV1 and Nav1.8) important for electrical activity in primary nociceptors by antisense knockdown or pharmacological inhibition (AMG9810). Evidence that chronic activity in primary nociceptors contributes to neuropathic SCI pain, evidence that nociceptor activity drives central neuroinflammation in peripheral injury models, and increasing support for the idea that nociceptors function within a unified host defense system all suggest that spinal and systemic effects of SCI can activate nociceptor-mediated host defense responses that interact with complex central consequences of SCI to drive chronic pain. This broader view of SCI-induced neuroinflammation may aid in the identification of new targets for treating SCI pain.

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Neurobiology of Disease

Persistent Electrical Activity in Primary Nociceptors after Spinal Cord Injury Is Maintained by Scaffolded Adenylyl Cyclase and Protein Kinase A and Is Associated with Altered Adenylyl Cyclase Regulation

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Little is known about intracellular signaling mechanisms that persistently excite neurons in pain pathways. Persistent spontaneous activity (SA) generated in the cell bodies of primary nociceptors within dorsal root ganglia (DRG) has been found to make major contributions to chronic pain in a rat model of spinal cord injury (SCI) (Bedi et al., 2010; Yang et al., 2014). The occurrence of SCI-induced SA in a large fraction of DRG neurons and the persistence of this SA long after dissociation of the neurons provide an opportunity to define intrinsic cell signaling mechanisms that chronically drive SA in pain pathways. The present study demonstrates that SCI-induced SA requires continuing activity of adenylyl cyclase (AC) and cAMP-dependent protein kinase (PKA), as well as a scaffolded complex containing AC5/6, A-kinase anchoring protein 150 (AKAP150), and PKA. SCI caused a small but significant increase in the expression of AKAP150 but not other AKAPs. DRG membranes isolated from SCI animals revealed a novel alteration in the regulation of AC. AC activity stimulated by Ca^{2+}-calmodulin increased, while the inhibition of AC activity by Gαi showed an unexpected and dramatic decrease after SCI. Localized enhancement of the activity of AC within scaffolded complexes containing PKA is likely to contribute to chronic pathophysiological consequences of SCI, including pain, that are promoted by persistent hyperactivity in DRG neurons.

Key words: A-kinase anchoring protein; chronic pain; cAMP; DRG; hyperexcitability; spontaneous activity

Significance Statement

Chronic neuropathic pain is a major clinical problem with poorly understood mechanisms and inadequate treatments. Recent findings indicate that chronic pain in a rat SCI model depends upon hyperactivity in dorsal root ganglia (DRG) neurons. Although cAMP signaling is involved in many forms of neural plasticity, including hypersensitivity of nociceptors in the presence of inflammatory mediators, our finding that continuing cAMP-PKA signaling is required for persistent SA months after SCI and long after isolation of nociceptors is surprising. The dependence of ongoing SA upon AKAP150 and AC5/6 was unknown. The discovery of a dramatic decrease in Gαi inhibition of AC activity after SCI is novel for any physiological system and potentially has broad implications for understanding chronic pain mechanisms.

Introduction

The mechanisms that maintain chronic pain after injury to either the peripheral or CNS are poorly understood, and these neuropathic pain mechanisms are inadequately targeted by available therapeutics (Cohen and Mao, 2014). Permanent, intractable pain is often produced by spinal cord injury (SCI) (Finnerup, 2013; Walters, 2014). Surprisingly, persistent pain-related behavior that develops in rats after contusive SCI at the thoracic level

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requires chronic, ongoing electrical activity in primary afferent neurons. This was shown by the reversal of behavioral measures of spontaneous and evoked pain produced by knocking down a voltage-gated Na^+ channel, Nav1.8, which is uniquely expressed in primary afferent neurons and required for nociceptor SA (Yang et al., 2014). SCI-induced chronic SA is especially prominent in the somata of C-fiber dorsal root ganglia (DRG) neurons, where it has been observed both *in vivo* and in small dissociated DRG neurons months after injury (Bedi et al., 2010). Most of the DRG neurons with SA after SCI are nociceptors as indicated by their small size and frequent expression of TRPV1 and Nav1.8 channels (Bedi et al., 2010; Wu et al., 2013; Yang et al., 2014). Given the strong evidence that SA generated in the somata of nociceptors contributes to chronic pain after SCI, it is important to define the mechanisms that persistently maintain this SA.

Persistent activity in the adenylyl cyclase (AC)-cAMP-protein kinase A (PKA) pathway has been suggested as a mechanism to maintain hyperexcitability in nociceptors for days or weeks after peripheral inflammation or injury (Aley and Levine, 1999; Liao et al., 1999; Song et al., 2006; Villarreal et al., 2009), but whether ongoing cAMP signaling contributes to pain-related hyperexcitability lasting months or longer in neuropathic conditions is unknown. Moreover, possible roles for macromolecular complexes that coordinate cAMP-dependent events, the nature of complexes that might be linked to nociceptor SA, and whether molecular regulation within the complexes is altered in nociceptors during any chronic pain conditions are unknown. Taking advantage of the fact that the SCI-induced SA that has been linked to chronic pain is retained in nociceptor somata after dissociation (Bedi et al., 2010; Wu et al., 2013; Yang et al., 2014), we demonstrate that chronic SA in nociceptors requires continuing activity of AC and PKA, plus the presence of an intact complex of AC5/6, PKA, and the scaffolding molecule A-kinase anchoring protein 150 (AKAP150, also called AKAP79 in humans or AKAP5). These findings plus novel alterations found in the regulation of AC after SCI provide new insights into basic mechanisms that maintain SA in nociceptors and the consequent excitation of pain pathways.

Materials and Methods

All procedures complied with guidelines of the International Association for the Study of Pain and were approved by the institutional animal care and use committee. Male rats (200–300 g) were maintained under a 12:12 h reversed light/dark cycle, and experiments were performed during the dark phase (Bedi et al., 2010).

SCI procedures. Contusion injury and postsurgical care were conducted as described previously (Bedi et al., 2010). Briefly, rats in the SCI group were deeply anesthetized with ketamine (80 mg/kg), xylazine (20 mg/kg), and acepromazine (0.75 mg/kg) before laminectomy of T10 vertebrae followed by a spinal impact using an Infinite Horizon impactor (150 kdyne, 1 s dwell time). Animals in the Sham group received identical surgery without spinal impact. Animals in the Naive group received no surgery. All SCI animals exhibited Basso, Beattie, and Bresnahan (BBB) hindlimb motor scores of 0–1 (Basso et al., 1995) 1 d after SCI. Hindlimb motor function showed only partial recovery when examined before excision of DRGs 1–6 months after SCI, similar to that described previously (Bedi et al., 2010; Yang et al., 2014).

Dissociation and culture of DRG neurons. Selected DRGs (L4, L5) were minced and incubated for 40 min at 34°C with trypsin (0.3 mg/ml) and collagenase D (1.5 mg/ml). DRG fragments were triturated, the neurons were plated without serum or growth factors onto 8 mm glass coverslips coated with poly-L-ornithine, and kept overnight in DMEM <5% CO_2, 95% humidity at 37°C.

Recording from dissociated DRG neurons. Whole-cell patch recordings of SA were made at ~23°C from small neurons (soma diameter ≤30 μm and input capacitance ≤45 pF) 18–28 h after dissociation using a MultiClamp 700B amplifier (Molecular Devices) as described previously (Bedi et al., 2010; Wu et al., 2013). Patch pipettes were pulled from borosilicate glass capillaries with a P-97 puller (Sutter) and fire polished to achieve a final electrode resistance of 3–8 MΩ. Pipettes were filled with solution containing the following (in mM): 134 KCl, 1.6 $MgCl_2$, 13.2 NaCl, 3 EGTA, 9 HEPES, 1 Mg-ATP, and 0.3 Na-GTP (pH 7.2 adjusted with KOH, 300 mOsM, adjusted with sucrose). The bath solution contained 140 NaCl, 3 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 10 glucose (pH 7.4 adjusted with NaOH, osmolarity 320 mOsM). After forming a tight seal (>1 GΩ), the membrane was ruptured. After whole-cell configuration was established under voltage clamp, the input capacitance was measured, and then capacitance and series resistance were electronically compensated. The calculated liquid junction potential was 4.3 mV and was not corrected. Spontaneous activity (SA) was measured in current-clamp mode, beginning 2 min after membrane rupture. In previous studies, we defined SA as at least 1 spike occurring within 60 s (i.e., minimum firing rate of 0.02 Hz) (Bedi et al., 2010). Because a demonstration of inhibitory effects on SA is impractical in cells with very low firing rates, in the current study we selected for neurons having initial SA firing rates of 0.1–5 Hz. For *in vitro* superfusion of DRG neurons with inhibitory or inactive control agents, solutions were driven by gravity from a set of independent tubes connected to an array of fused silica columns (inner diameter, 200 μm). Rapid exchange of solutions was achieved by shifting the columns horizontally with a micromanipulator. The distance from the column mouth to the recorded cell was ~100 μm. A subset of neurons was recorded with the perforated patch method to achieve stable recordings for much longer periods. Recordings were made with gramicidin (50 μg/ml) in the recording pipette following established procedures (Wu et al., 2013; Zhu et al., 2014). The PKA inhibitors, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), Rp-cAMPs, and the AC inhibitor 2′deoxyadenosine 3′-monophosphate (2′deoxy-3′AMP) were purchased from Sigma-Aldrich. The AKAP disrupting peptide stHt31 and its control stHt31P were purchased from Promega. Electrophysiological data were analyzed using PatchMaster, Clampfit version 10.4 (Molecular Devices) and Origin 5.0 (MicroCal Software).

Antibodies used for immunoprecipitation and Western blotting. These were mouse or rabbit anti-AKAP150 (EMD Millipore for Western blotting and Santa Cruz Biotechnology for immunoprecipitation) (Hoshi et al., 2003), mouse anti-AKAP12 (Sigma-Aldrich, clone JP74) (Havekes et al., 2012), mouse anti-PKA RIIβ (BD Transduction Laboratories) (Hoshi et al., 2003), mouse anti-β-actin (Santa Cruz Biotechnology), and normal mouse or rabbit IgG (Santa Cruz Biotechnology). The mouse anti-AC5 hybridoma cell lines were generated by Genscript against human AC5 peptide CGNQVSKEMKRMGFEDPKDKN (Hu et al., 2009). Antibodies were purified by affinity chromatography using antigen immobilized peptide and are highly selective for AC5 over AC6 (data not shown).

RT-PCR of AC isoforms. Fresh DRGs or RNA later-stabilized DRGs were homogenized on ice using a conventional rotor-stator homogenizer (RNase-free). Total RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the protocol suggested by the manufacturer. The RNA was eluted with RNase-free water and quantitated using the NanoDrop 1000 spectrophotometer. First-strand cDNA was generated from DNase-treated total RNA (250 ng-600 ng) using Moloney murine leukemia virus reverse transcriptase (New England Biolabs) and oligo(dT) primers. qRT-PCR was performed using an Eppendorf Mastercycler ep *realplex*^{2} (Eppendorf) in the presence of SYBR Green I. Primers to rat AC isoforms 1–9 (Table 1) and rat GAPDH as the control were designed with NCBI Primer-Blast or using published sequences (Bek et al., 2001; Landa et al., 2005). The 10 ng cDNA template was used in each reaction, performed in triplicate for each primer set. The cycling conditions were as follows: 2 min at 94°C followed by 40 cycles of 15 s at 94°C, 0.5 min at 60°C, and 0.5 min at 72°C. The fold change in transcript levels ($2^{\Delta\Delta Ct}$) between SCI and Sham control samples was calculated from the cycle times (C_t) of each primer set as follows, where $\Delta\Delta Ct = \Delta Ct(ACX_{SCI} - GAPDH_{SCI}) - \Delta ct(ACX_{Sham} - GAPDH_{Sham})$. Statistical analysis was performed according to Rieu and Powers (2009). Amplified PCR products were run on 1.5% agarose gels after 35–40 cycles. Products

Table 1. Primers to rat AC isoforms 1–9

AC isoform	Primer	Sequence	Position GenBank	Product size (bp)
AC1	Sense	5′-TCCCCGGAACATGGATCTCT-3′	2415-2434	481
	Antisense	5′-CCCAGTGCTATCCATCCGAC-3′	2895-2876	
AC2	Sense	5′-CTCCATTCCGGACTTCAAGG-3′	2739-2758	268
	Antisense	5′-CAGGGCATATGCAAACTCCA-3′	3006-2987	
AC3	Sense	5′-GTCACCTTAGTCACGAGAGC-3′	4359-4378	184
	Antisense	5′-AGTAGCAAAGGCGACTGTAG-3′	4542-4523	
AC4	Sense	5′-GGAAGACGAGAAGGGCACCGAGAG-3′	1502-1525	467
	Antisense	5′-GAGCTGGGGGCCTGGTTGTCAC-3′	1968-1947	
AC5	Sense	5′-ACCAAGGCCACACTCAACTAC-3′	2255-2275	163
	Antisense	5′-GGTTCATCTTGGCGATCA-3′	2417-2400	
AC6	Sense	5′-CAAAGGAAGGGACGCCGAGAGG-3′	3731-3752	391
	Antisense	5′-GGCAAATGGAAACATTCCCCTG-3′	4122-4101	
AC7	Sense	5′-CGTTCCCGGACTTCAAAGTGT-3′	2712-2731	355
	Antisense	5′-AATCACTCCAGCAATCACAGG-3′	3066-3046	
AC8	Sense	5′-CAGTCTGGGCCTGAGGAAATT-3′	2748-2768	478
	Antisense	5′-AAGTCAGGTTCTTCAAGGGTA-3′	3225-3205	
AC8′	Sense	5′-TTCACTTGAGCCTAGCCTCG-3′	1363-1382	627
	Antisense	5′-GGATGTAGATGCGGTGGAAC-3′	1989-1970	
AC9	Sense	5′-CGGTCTCCCACAGATGAGAT-3′	3856-3875	351
	Antisense	5′-TCTGGGGACAGAAACTGAGG-3′	4206-4187	
GAPDH	Sense	5′-TATGACAACTCCCTCAAGAT-3′	483-502	317
	Antisense	5′-AGATCCACAACGGATACATT-3′	799-780	

for AC6 and AC8 were detected by agarose gels upon longer cycles and also with alternate primers (AC8′); AC1 and AC4 product levels were very low, and AC7 was below the level of detection for qRT-PCR. All primers were confirmed using rat brain tissue.

Immunoprecipitation of AC activity. DRGs below the vertebral T10 injury level from sham or SCI rats were used for all biochemical assays. Previous work showed similarly high incidence of SA after SCI in small neurons from DRGs from just above the injury level (T8, T9), at the injury level (T10, T11, T12), and below the injury level (L3 to L6) (Bedi et al., 2010; unpublished observations). Thus, to maximize the amount of relevant DRG tissue, we pooled DRGs bilaterally from T12 to L6. Immunoprecipitation of protein complexes with rabbit IgG (control) or anti-AKAP150 antibodies was performed as described previously (Piggott et al., 2008; Kapiloff et al., 2009; Efendiev et al., 2013). Disrupting peptides (10–20 μM) were added during homogenization and were prepared as described previously (Efendiev et al., 2010). Immunoprecipitations were assayed for associated AC activity upon stimulation with 100 nM GTPγS-Gαs and 50 μM forskolin for 10 min at 37°C. AC8 activity was measured upon stimulation with 100 μM Ca^{2+} and 300 nM calmodulin. Acetylated cAMP was detected by enzyme immunoassay (ELISA kit from Enzo Life Sciences).

AC activity in DRG membranes. Freshly isolated DRGs were rinsed with PBS and resuspended in sucrose supplemented buffer: 20 mM HEPES, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 250 mM sucrose, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*α-p-tosyl-L-lysine chloromethyl ketone, and 0.1 mM *N*-tosyl-L-phenyl alanine chloromethyl ketone). Tissue was homogenized using a rotor-stator tissue homogenizer (5 mm probe; 3 s pulses on ice, 7 times) and centrifuged at 1800 × *g* to pellet nuclei. Membranes were subjected to centrifugation for 20 min at 60,000 × *g*, resuspended in sucrose supplemented buffer, and analyzed for protein concentration by Bradford assay. Membranes (6–10 μg/reaction) were immediately assayed for AC activity upon stimulation with the indicated reagents (Dessauer, 2002). Heart membranes were prepared from Naive/Sham or SCI animals as described above for DRGs, except that flash frozen tissue was rapidly thawed and quartered before homogenization. Membranes from Sf9 cells expressing AC1 or AC5 were prepared as described previously (Dessauer, 2002).

Statistical analysis. Data are presented as mean ± SEM. Comparisons were made with paired or unpaired *t* tests, one-way ANOVA followed by Bonferroni's *post hoc* tests corrected for multiple comparisons, or two-way ANOVA with repeated measures. SA incidence was compared using Fisher's exact tests. In many of the biochemical studies, specific a priori predictions based on published or unpublished pilot studies were tested between selected pairs of groups using *t* tests. Each group used for biochemical comparisons included at least three experiments, with each experiment performed in duplicate or triplicate. Statistical analyses were performed with Prism 6 (Graphpad Software).

Results

Activity of anchored PKA is required for persistent SCI-induced SA in DRG neuron somata

We first asked whether continuing PKA activity is required for the maintenance of SCI-induced SA in small DRG neurons. SA occurs in the somata of numerous nociceptors at and below the injury level for at least half a year after thoracic spinal contusion, and its incidence is correlated with pain-related behavior (Bedi et al., 2010). Importantly, SCI-induced nociceptor SA is abolished by interventions that reverse behavioral signs of pain (Wu et al., 2013; Yang et al., 2014). As we found previously, SCI significantly increased the incidence of L4/L5 neurons with SA (Fig. 1*A*) compared with neurons in Naive or Sham groups (Fig. 1*B*, top bars): increased SA was found both 3 d after SCI (SA in 11 of 14 neurons, 79%) and 1–6 months after SCI (23 of 32 neurons, 72%). No significant differences in SA incidence were found between neurons tested 3 d versus 1–6 months after SCI in any of the SCI groups, regardless of treatment after dissociation, so early and late test data were combined for each group (more than half of each group shown was tested 1–6 months after SCI). The increase in SA incidence after SCI was associated with significant depolarization of resting membrane potential (RMP) (Fig. 1*B*, bottom bars) (see also Bedi et al., 2010).

Previous work implicated ongoing PKA activity in the maintenance of hyperexcitability of small DRG neuron cell bodies (Song et al., 2006; Zheng et al., 2007) and in the induction of one type of hyperalgesic priming (Araldi et al., 2015a,b). We found that bath application of Rp-cAMPS (100 μM, applied at least 30 min before establishing whole-cell configuration) significantly decreased the incidence of SA in DRG neurons dissociated from DRG in SCI animals, and tended to hyperpolarize RMP (Fig. 1*A*,*B*). To determine whether a PKA inhibitor would also reduce ongoing SA from a dissociated DRG neuron, we superfused a more membrane-permeant inhibitor, H-89 (50 μM), over individual cells beginning 2–4 min after establishing whole-cell configuration. As shown in Figure 1, *C* and *D*, spontaneous discharge in the small DRG neurons we sampled was irregular and showed considerable variability within and between cells (see also Bedi et al., 2010). SA firing rates tended to increase during the first minute of whole-cell recording and then stabilize. H-89 applied when firing had stabilized ($n = 6$) produced a decrease in firing rate within 40 s, and SA was essentially eliminated after 2 min of superfusion. Little or no recovery occurred within 3–5 min of washout ($n = 4$, data not shown). Two-way ANOVA with repeated measures revealed significant effects of time after superfusion ($F_{(12,168)} = 2.53$; $p = 0.0044$), 50 μM H-89 treatment ($F_{(1,14)} = 5.51$; $p = 0.0341$), and their interaction ($F_{(12,168)} = 2.59$; $p = 0.0035$). Identical experiments using 10 μM H-89 under whole-cell patch recording conditions ($n = 3$) failed to reduce SA within 5 min (data not shown). Because membrane-permeant PKA antagonists such as H-89 are typically bath applied at least 10 min before electrophysiological recording, and it is difficult to maintain cell health for 10 min or more with the whole-cell patch configuration, we used the much less damaging (but more technically difficult) perforated patch method to test the possibility that the lower 10 μM concentration of H-89 would suppress SA if allowed more time to penetrate into the cell. In small DRG neu-

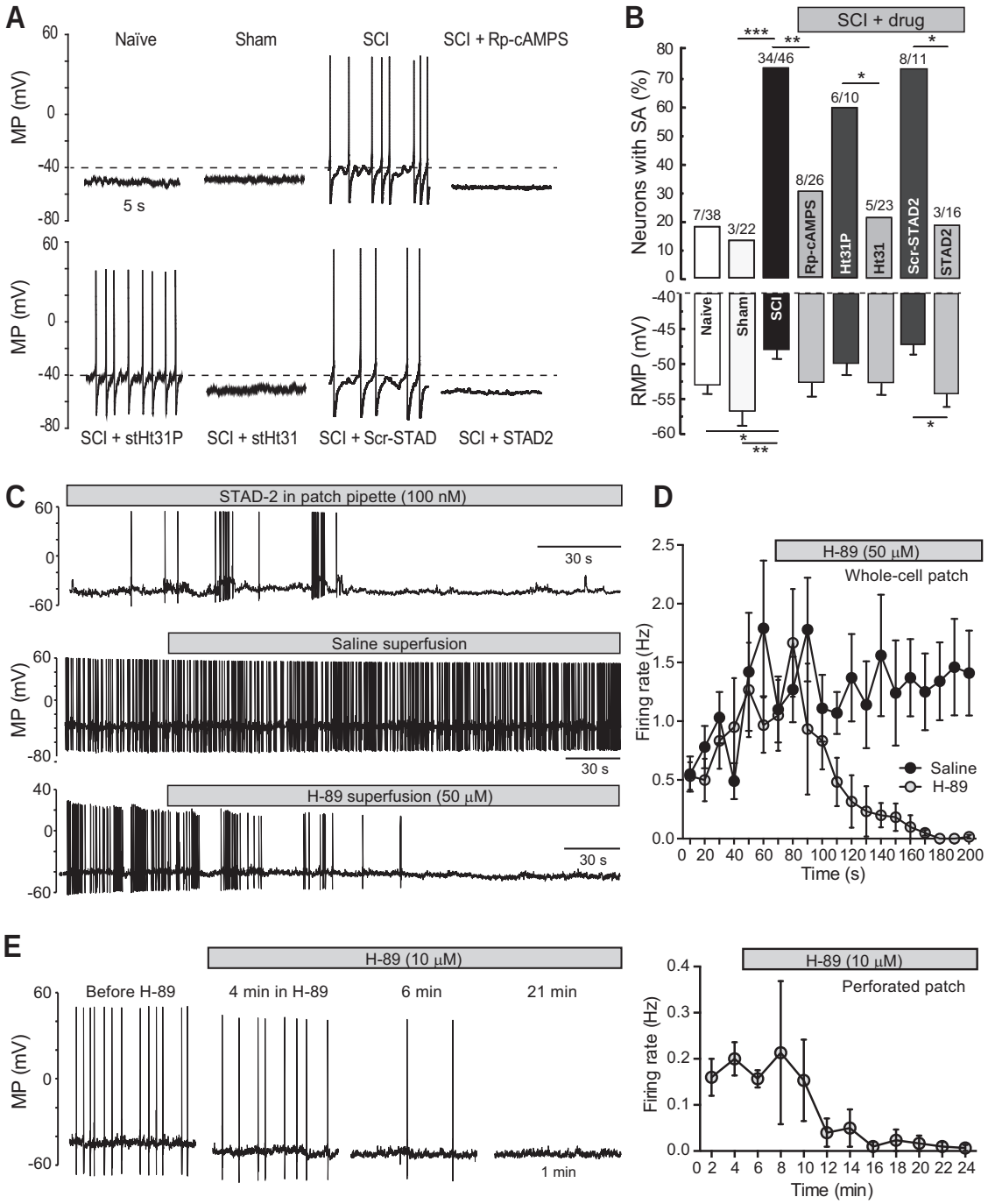

Figure 1. Anchored PKA activity maintains SCI-induced SA in dissociated small L4/L5 DRG neurons. ***A***, Examples of SA and RMP after the indicated treatments. Dashed line indicates least negative acceptable RMP (−40 mV) for sampled neurons. ***B***, Attenuation of SCI-induced SA and depolarized RMP by inhibitors of PKA and AKAP function. The ratio above each bar denotes the number of neurons with SA/the number of neurons sampled. Statistical comparisons of SA incidence were made with Fisher's exact tests on the indicated pairs of groups (paired experiments were run on the same batches of neurons in different coverslips on the same day). Comparisons of RMP (mean ± SEM) were made with one-way ANOVA followed by Bonferroni *post hoc* tests: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. ***C***, Examples of ongoing SA in dissociated DRG neurons and its rapid reduction by inhibitors of PKA and AKAP function. Top, Cell recorded under whole-cell configuration with a higher resistance patch pipette containing 100 nM STAD-2 in pipette solution. Middle, Cell recorded with lower resistance patch pipette and superfused with saline (same as bath solution). Bottom, Cell recorded with a lower resistance pipette and superfused with H-89 in bath solution. ***D***, Time course of the effects of superfused 50 μM H-89 on SA firing rate (mean ± SEM). Two-way ANOVA revealed significant effects of H-89 treatment ($n = 6$ neurons) versus controls ($n = 10$) and of time after treatment. ***E***, Prolonged recordings of dissociated DRG neurons with the perforated patch method shows that 10 μM H-89 eventually suppresses ongoing SA. Left, Examples of SA recorded 1 min before and at the indicated times after superfusion of 10 μM H-89. Right, Time course of the effects of 10 μM H-89 treatment on mean SA firing rate recorded with perforated patch. Each point represents the mean ± SEM ($n = 3$ cells) of the average spike frequency in each cell during the indicated 2 min period. MP, Membrane potential (includes action potentials).

rons showing stable perforated patch recordings for long periods (25–75 min, $n = 3$), 10 μM H-89 produced clear, prolonged suppression of SA within 5–10 min of extracellular application (Fig. 1*E*).

PKA is often targeted to signaling complexes by specific binding to AKAPs. We tested the necessity of this interaction by using a stearated AKAP-PKA disrupting peptide, stHt31, to inhibit the interaction between AKAPs and the regulatory subunits of PKA.

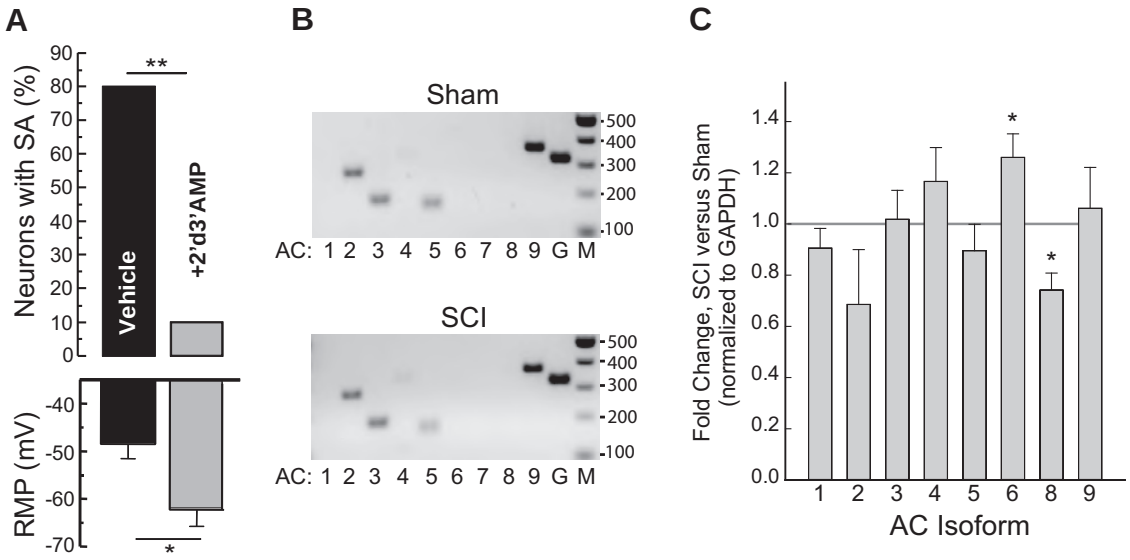

Figure 2. AC activity maintains SCI-induced SA. ***A***, Attenuation of SCI-induced SA and depolarized RMP by an AC inhibitor (2′deoxy-3′AMP). The proportion of neurons exhibiting SA was analyzed with Fisher's exact test (**$p < 0.01$; 10 cells per group); RMP analyzed with unpaired *t* test (*$p < 0.05$). ***B***, Agarose gel of RT-PCR products from RNA isolated from Sham and SCI rats of AC isoforms and GAPDH control after 40 cycles. Primers used are indicated in Table 1. ***C***, qRT-PCR of AC isoform expression in DRGs from Sham and SCI rats. Fold change ($2^{-\Delta\Delta Ct}$) after SCI ± SD is plotted. All samples were normalized to GAPDH controls ($n = 3$, performed in triplicate). For description of statistical analysis, see Materials and Methods.

Intracellular dialysis of stHt31 (50 μM) through the pipette (in the whole-cell patch configuration) into neurons isolated from SCI animals significantly reduced the incidence of neurons displaying SA, whereas an inactive version of this peptide, stHt31P (50 μM), had no effect (Fig. 1*A*,*B*). As a complementary approach, we used STAD-2, a stable "hydrocarbon-stapled" PKA-AKAP disrupting peptide that is highly selective for the RII regulatory subunit of PKA (Wang et al., 2014). Intracellular delivery of STAD-2 (500 nM) significantly reduced SA incidence and hyperpolarized the RMP, compared with effects of the scrambled inactive version, Scr-STAD (Fig. 1*A*,*B*).

For both stHt31 and STAD-2, the inhibitory effects were very rapid. The cells lacking SA exhibited no SA at any point during the recording session, even at the onset (30–60 s after establishing whole-cell configuration). Moreover, the few cells with SA did not show a progressive decrease in firing rate after breaking into the cell. These observations suggest that, at these concentrations and with the 30–60 s delay between break-in and recording onset, the inhibitors had an all-or-none effect on recorded SA. To observe the development of the predicted inhibition of ongoing SA, we used a lower concentration of inhibitor applied through a smaller aperture patch pipette. We found 4 neurons that exhibited SA when recordings began 15–30 s after break-in using a patch pipette containing 100 nM STAD-2 (instead of 500 nM) and having a resistance of 7–12 MΩ (instead of 3–6 MΩ). In each case, the early SA was abolished within 100 s of break-in, as illustrated in Figure 1*C*, and no further SA occurred for the remainder of the recordings (2–10 min of inactivity). These results confirm that ongoing SA observed in small dissociated DRG neurons after SCI is blocked by disruption of AKAP-PKA interactions.

Interestingly, even at the higher concentrations used in most of our recordings, neither STAD-2 nor stHT31 completely eliminated SA in the sampled populations. Indeed, the SA incidence in the presence of these inhibitors was about the same as in cells sampled from Naive and Sham groups (Fig. 1*B*). Moreover, neither inhibitor caused the DRG neurons to become inexcitable; artificial injection of depolarizing current in the presence of either inhibitor was still able to evoke action potentials (data not shown). Together, these results indicate that ongoing activity of PKA anchored by the RII subunit to an AKAP is continuously required for the generation of persistent SA in isolated nociceptors induced by prior SCI, but this activity is not required for normal excitability.

AC5/6 complexed with AKAP150 is required for persistent SCI-induced SA

The requirement of anchored PKA activity for persistent SCI-induced SA implied that AC activity is also necessary. Using intracellular dialysis of the AC P-site inhibitor, 2′deoxy-3′AMP (300 μM), we found significant reduction of SA incidence and significant hyperpolarization in small sensory neurons from SCI rats compared with neurons dialyzed with vehicle (Fig. 2*A*). To obtain a complete profile of AC isoform expression, we used qRT-PCR to compare DRG transcript levels between Sham and SCI groups. Largely in accord with previous studies (Ehnert et al., 2004), AC2, AC3, AC5, and AC9 were detected in DRGs taken from T12 to L6 segments and, to a lesser extent, AC6 and AC8 (Fig. 2*B*). Relatively small differences in RNA levels for any AC isoform were found between SCI and Sham groups, although AC6 was significantly elevated and AC8 was significantly reduced in the SCI preparations (Fig. 2*C*).

Most small DRG neurons that exhibit SA after SCI express TRPV1 (Wu et al., 2013). In nociceptors, TRPV1 is complexed with AKAP150 and AC5 and/or AC6 (Efendiev et al., 2013). Therefore, we probed this complex using an AC-AKAP150 disrupting peptide that is selective for the AC5/6 binding domain of AKAP150 (AKAP79$^{77-153}$, hereafter called AC-AKAPi) (Efendiev et al., 2010, 2013). We have previously shown that AC-AKAPi is selective for AC5/6 and does not displace AC2 or AC9 from the AKAP79/150 scaffold (Efendiev et al., 2010). AC3 and AC8 activity can also be immunoprecipitated with AKAP150 when overexpressed in HEK293 cells (Fig. 3*A*) (Efendiev et al., 2010). AC-AKAPi (10 μM) had no effect on AKAP150 anchoring of AC3 or AC8 but disrupted AC5 interactions with the scaffold (Fig.

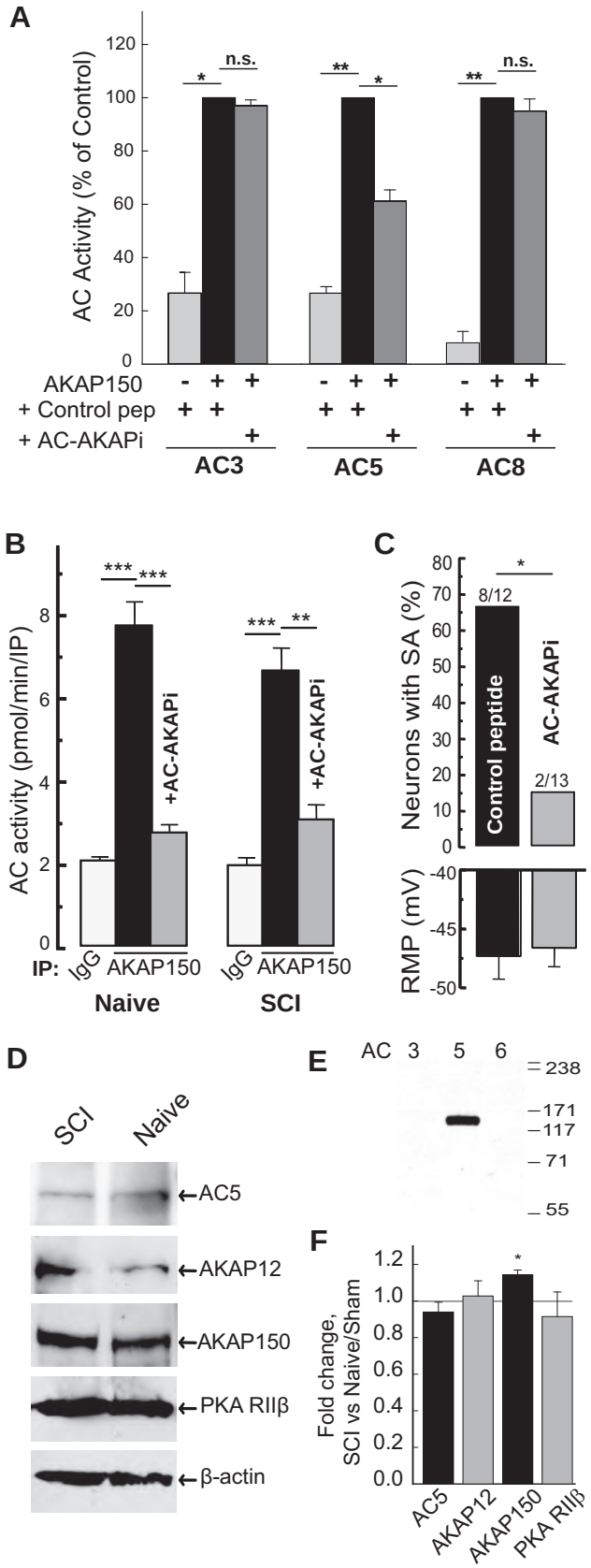

Figure 3. Disruption of AKAP150-anchored AC in DRGs by an AC5/6-AKAP150-selective disrupting peptide (AC-AKAPi) attenuates SCI-induced SA. ***A***, AC-AKAPi is selective for AC5 and does not disrupt association of AC3 or AC8 with AKAP150. HEK293 cells were transfected with AKAP150 and the indicated AC isoforms. Samples were immunoprecipitated with anti-AKAP150 in the presence of control or disrupting peptides (10 μM) during cell lysis, and the associated stimulated AC activity was measured (mean ± SEM, $n = 3$ performed in duplicate, normalized to control peptide with AKAP150). *$p < 0.05$ (paired t test on non-normalized data). **$p < 0.01$ (paired t test on non-normalized data). ***B***, AKAP150-associated AC activity is reduced by AC-AKAPi in DRGs from sham and SCI rats. Samples were immunoprecipitated with control IgG or anti-AKAP150 in the presence or absence of AC-AKAPi (20 μM), and associated AC activity was measured (mean ± SEM, $n = 3$). ***$p < 0.001$ (unpaired t test). ***C***, Attenuation of SCI-induced SA by AC-AKAPi. *$p < 0.05$ (Fisher's exact test). ***D***, ***F***, AKAP150, but not AC5, protein expression in DRGs is slightly enhanced by SCI (mean ± SEM, $n = 3$–5). *$p < 0.05$ (paired t test of SCI vs Sham controls). ***E***, Characterization of anti-AC5. WB of membranes from Sf9 cells expressing AC3, AC5, or AC6. n.s., Not significant.

3*A*). Our control peptide (AKAP79$^{109-290}$) had no effect on the association of any AC isoform with AKAP79/150 (Fig. 3*A*) (Efendiev et al., 2010). To determine the level of AC activity associated with AKAP150 in SCI and Naive animals, we immunoprecipitated AKAP150, using IgG as a control, from lysates of DRGs taken from segments T12 to L6 followed by Gαs/forskolin-stimulated AC activity measurements. Antibodies against AKAP150 pulled down nearly fourfold increased AC activity compared with IgG in preparations from both SCI and Naive groups (Fig. 3*B*). The disrupting peptide AC-AKAPi (20 μM) significantly reduced AKAP150-associated AC activity obtained from both Naive and SCI groups (Fig. 3*B*). As AC-AKAPi is selective for AC5 and AC6, and does not affect interactions of AKAP150 with other AC isoforms (Fig. 3*A*) (Efendiev et al., 2010), these results indicate that most of the AC activity associated with AKAP150 in DRGs is produced by AC5/6.

To investigate the requirement of this complex for the maintenance of SCI-induced SA, we dialyzed small sensory neurons isolated from SCI rats with AC-AKAPi (20 μM) or a control peptide, AKAP150$^{109-290}$ (20 μM), that does not affect AC-AKAP150 interactions. As found with the PKA-AKAP disrupting peptides, dialysis with AC-AKAPi caused a significant decrease in SA incidence compared with the control peptide (Fig. 3*C*), albeit with no apparent effect on RMP. This result strengthens the conclusion that AC5/6-AKAP150 interactions are required for persistent nociceptor SA.

Previous studies have identified expression of several AKAPs in nociceptors, including AKAP150, AKAP12 (also known as Gravin), AKAP18, and Yotiao (Schnizler et al., 2008). Western blotting confirmed expression of AKAP150, AKAP12, and the RIIβ subunit of PKA in DRGs, but the protein levels were largely unchanged after SCI (Fig. 3*D*). A small but significant increase in AKAP150 was detected after SCI. AC5 protein levels were also unchanged (Fig. 3*D*); limited amounts of DRG tissue and inadequate quality of antibodies to other AC isoforms prevented detection of other AC isoforms.

SCI alters the regulation of ACs in DRGs

An important question is whether SCI alters the function of the AC5/6-AKAP150-PKA complex. A persistent functional change in this complex could be important for driving chronic SA and consequent pain. To begin to address this question, we looked for SCI-induced modifications in the regulation of AC activity in membranes isolated from DRGs taken from T12 to L6 segments in control (Naive and Sham) and SCI rats. AC activity measurements were made under basal enzyme conditions, during stimulation with purified GTPγS-bound Gαs (100 nM) either in the presence or absence of GTPγS-Gαi (1 μM), and upon stimulation with Ca^{2+}/calmodulin (100 μM/300 nM; Fig. 4*A*). Membranes from Naive ($n = 2$) and Sham ($n = 2$) rats showed identical response patterns, so these control data were pooled. Previous

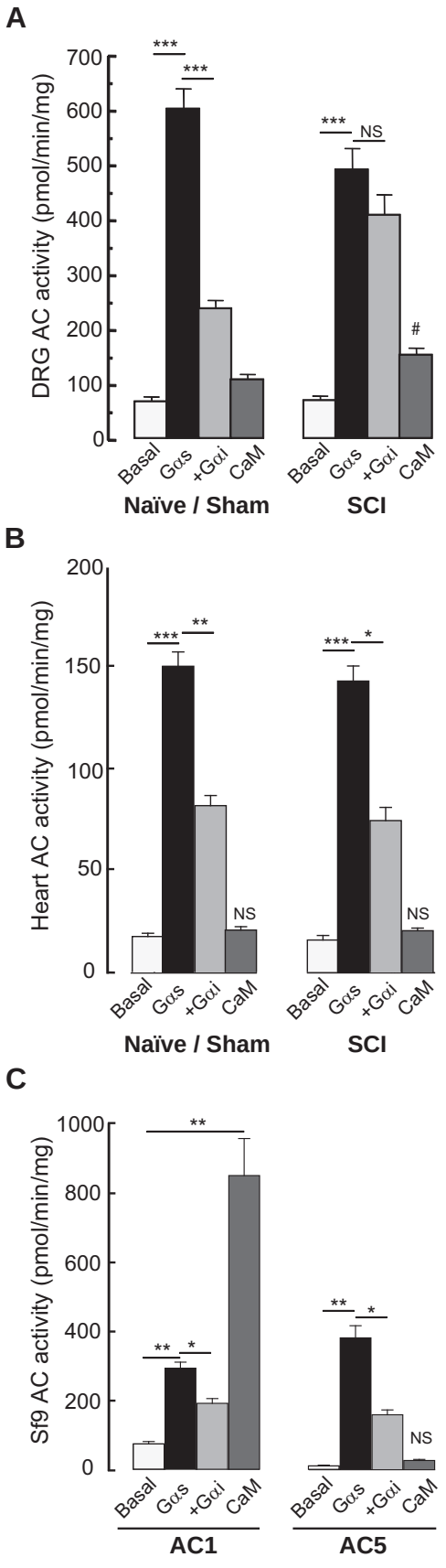

Figure 4. SCI reduces Gαi inhibition of AC activity in DRG membranes and increases Ca^{2+}/calmodulin-stimulated activity without changing basal or total Gαs-stimulated AC activity. AC activity was measured in membranes prepared from (***A***) DRGs ($n = 4$), (***B***) heart ($n = 3$), or (***C***) Sf9 cells expressing AC1 or AC5 ($n = 3$) under basal conditions or in the presence of Gαs (100 nM), Gαs plus Gαi (1 μM), or Ca^{2+}/calmodulin (CaM; 100 μM/300 nM). $^{*}p < 0.05$ (unpaired t tests). $^{**}p < 0.01$ (unpaired t tests). $^{***}p < 0.001$ (unpaired t tests). $^{\#}$Significant difference in CaM-stimulated AC responses of SCI versus Naive/Sham animals. NS, Not significant.

studies have failed to find significant differences in nociceptor physiology or pain-related behavior between Naive and Sham animals (Bedi et al., 2010; Wu et al., 2013; Yang et al., 2014). Gαs produced an eightfold increase in activity, which was inhibited 60% by the presence of Gαi. This pattern indicates major contributions from AC5/6, which are activated by Gαs, inhibited by Gαi, and unaffected by Ca^{2+}/calmodulin. Stimulation by Ca^{2+}/calmodulin alone caused a 1.5-fold enhancement of basal AC activity, suggesting weak contributions from AC1, AC3, and/or AC8.

Importantly, DRG membranes isolated from SCI animals ($n = 4$) showed significant differences in AC regulation compared with membranes from the control animals; there was a dramatic loss of Gαi inhibition and a 1.5-fold increase in AC activity stimulated by Ca^{2+}/calmodulin (Fig. 4*A*). SCI had no significant effect on the total basal or Gαs-stimulated AC activity. This alteration in AC regulation after SCI was not a generalized effect of SCI, as no alterations in Gαi inhibition or Ca^{2+}/calmodulin stimulation were detected in heart tissue from SCI groups compared with Naive/Sham groups (Fig. 4*B*). Examples of AC regulatory patterns are shown as controls in Figure 4*C*, where membranes from Sf9 cells expressing AC1 or AC5 were assayed under the same regulatory conditions as above. Although both AC isoforms were inhibited by Gαi (35% and 58% for AC1 and AC5, respectively), only AC1 (and AC8, data not shown) was activated by Ca^{2+}/calmodulin (10-fold). These findings suggest that SCI-induced alterations in the regulation of ACs, especially AC5/6, are important for driving PKA activity and promoting nociceptor SA.

Discussion

This study has revealed a previously unsuspected role for AC5 and/or AC6 complexed with AKAP150 and PKA in maintaining nociceptor hyperactivity after SCI, and has disclosed novel alterations of AC function that have not been reported previously in any physiological context.

Increases in cAMP synthesis in primary afferent neurons have long been known to participate in various responses to bodily injury, including axonal regeneration (Siddiq and Hannila, 2015) and sensitization (Gold and Gebhart, 2010). In nociceptors, increased cAMP synthesis contributes to sensitization by inflammatory signals lasting several hours (e.g., Taiwo et al., 1989; Cui and Nicol, 1995; Hingtgen et al., 1995; Kress et al., 1996; Gold et al., 1998), and the acute sensitization of nociceptors by heat or prostaglandin E_2 depends upon AKAP150-PKA complexes (Jeske et al., 2008; Schnizler et al., 2008; Efendiev et al., 2013; Fischer and McNaughton, 2014). Surprisingly few studies have investigated whether AC-PKA signaling also contributes to the maintenance of persistent hyperexcitability of nociceptors. Inhibitors of PKA activation or AC activity can reduce nociceptor hyperexcitability when applied days or weeks after nerve crush (Liao et al., 1999), peripheral prostaglandin E_2 injection (Aley and Levine, 1999; Villarreal et al., 2009), or "chronic" (1–2 weeks) compression of lumbar DRGs (CCD model) (Song et al., 2006; Zheng et al., 2007; Huang et al., 2012). Inhibition of PKA activity by H-89 has been reported to block the induction of hyperalgesic priming (lasting several weeks) by agonists of mu opioid receptors (Araldi et al.,

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2015a) or adenosine A1 receptors (Araldi et al., 2015b). However, in these priming models, PKA activity only appeared to contribute for several days to the maintenance of the primed state, and it is unknown whether the reported behavioral alterations are driven by nociceptor hyperexcitability or SA. In contrast, we found that inhibition of PKA activation by Rp-cAMPS, disruption of PKA anchoring to AKAPs, or inhibition of AC activity by 2′deoxy-3′AMP eliminated both the SA and depolarization of RMP in small DRG neurons observed months after SCI. In addition, the PKA inhibitor H-89 was found to rapidly abolish SA months after SCI. Dependence of nociceptor hyperexcitability on AC-cAMP-PKA signaling in the CCD model has been associated with increased expression of PKA, including RIIβ (Huang et al., 2012), which we did not find after SCI. A major difference between our SCI model and most neuropathic pain models, such as CCD, is that most of the sensory neurons tested in the SCI model are probably not damaged by the neuropathic insult (Bedi et al., 2010). Moreover, unlike in the CCD model (Huang et al., 2012), sensory neuron hyperexcitability recorded after SCI does not depend upon activation of protease-activated receptor 2 (Q. Yang and E. Walters, unpublished observations). The present study shows, for the first time in any pain model, that AC activity is necessary to maintain nociceptor hyperexcitability months after the initiating event. This demonstration is particularly noteworthy because the AC-dependent ongoing SA occurs in the same sensory neurons whose persistent hyperactivity has been shown to be necessary for behavioral signs of chronic pain after SCI (Yang et al., 2014).

The displacement of AC5/6 from the AKAP150 complex by a selective disrupting peptide (AC-AKAPi) strongly reduced SA, providing clear evidence that SCI-induced SA depends upon signaling activity in a complex containing AC5/6, PKA, and AKAP150, all of which are present in DRGs from naive and SCI rats. This finding is interesting in light of the dramatic deficits in behavioral measures of nociception and neuropathic pain found in AC5 null mutant mice (Kim et al., 2007). While those deficits were assumed to reflect loss of AC5 function in the CNS, the present results suggest that loss of AC5 function in primary nociceptors could have contributed to the results reported by Kim et al. (2007).

We found no increase in expression of PKA and only a small increase in expression of AKAP150 after SCI. In contrast, we found a dramatic change in the regulation of AC activity. Two regulatory alterations occurred after SCI. First, there was a ~50% increase in the relatively small amount of AC activity stimulated by Ca^{2+}/calmodulin. In principle, this could contribute to hyperexcitability and is consistent with either increased expression or increased sensitivity of AC1, AC3, or AC8. AC1 contributes to chronic pain mechanisms in the brain (Vadakkan et al., 2006) but was not detected in DRGs (Fig. 2*B*) (Ehnert et al., 2004). AC8 in the spinal cord and brain has been suggested to contribute to persistent pain (Wei et al., 2002) and binds AKAP150 (Efendiev et al., 2010; Willoughby et al., 2010), but AC8 would represent a small fraction of AKAP150-associated AC in DRGs, even after SCI. Importantly, the function of numerous AKAP150-anchored channels is known to be either enhanced (e.g., Cav1.2, Cav3.2, TRPV1, and TRPV4) or inhibited (KCNQ and TREK) by PKA (Fischer and McNaughton, 2014), and any of these or yet to be identified channels associated with AKAP150 may promote nociceptor SA.

Second and perhaps more important, there was a large and novel effect of SCI on the regulation of AC activity by Gαi: the strong inhibitory effect of Gαi on Gαs-stimulated AC activity observed in naive animals was nearly eliminated after SCI. This loss of inhibition also is likely to contribute *in vivo* to increased AC activity and maintenance of nociceptor SA. The altered regulation of ACs in DRG membranes after SCI differs from the sensitization of AC5/6 that occurs following prolonged stimulation of $G\alpha_{i/o}$-coupled receptors by opioids (Watts and Neve, 2005) because we found no increase in total Gαs-stimulated activity. The altered regulation occurs at the level of the AC, and thus differs from the Gαi-dependent nociceptor mechanism proposed for chronic hyperalgesia in which stress or inflammation may switch the coupling of some receptors from Gαs (leading to PKA activation) to Gαi (leading to PKCε activation) (Khasar et al., 2008). Instead, SCI causes a loss of Gαi inhibition of AC activity. This might, in principle, be caused by a change in the relative protein expression of different AC isoforms. However, the only significant changes in relative mRNA levels for AC isoforms after SCI were an increase in AC6 and decrease in AC8. If these changes were translated into corresponding changes in protein levels, one would expect increased rather than decreased inhibition by Gαi, as AC6 is one of the AC isoforms most sensitive to Gαi inhibition (Taussig et al., 1994; Chen-Goodspeed et al., 2005). Instead, it is likely that SCI causes intrinsic modification of AC5/6 that reduces its inhibition by Gαi. An interesting question is whether, in addition to contributing to the maintenance of chronic nociceptor hyperactivity in conditions such as SCI, loss of Gαi inhibition of AC might contribute to the PKA-dependent ("Type II") hyperalgesic priming that can be produced by repeated peripheral stimulation of Gαi (Araldi et al., 2015a,b). The unexpected chronic alterations of AC regulation are consistent with SCI-induced reprogramming of numerous, uninjured nociceptors into a persistent, hyperactive cellular state that was proposed (Bedi et al., 2010; Walters, 2012) and then validated (Yang et al., 2014) as a major contributor to chronic pain. While the actual contributions of altered AC regulation to behavioral measures of pain after SCI (and potentially other pain conditions) have yet to be determined and the molecular mechanisms underlying the alteration of AC regulation remain to be defined, the present results provide the first direct demonstration that persistent hyperactivity of sensory neurons that excite pain pathways is associated with long-lasting alterations of AC function and regulation in the hyperactive neurons.

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